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(54) Title: RECOMBINANT VASCULAR ENDOTHELIAL CELL GROWTH FACTOR D (VEGF-D)

(57) Abstract

VEGF-D, a new member of the PDGF family of growth factors, which among other things stimulates endothelial cell proliferation and angiogenesis and increases vascular permeability, is described, as well as nucleotide sequences encoding it, methods for producing it, antibodies and other antagonists to it, transfected or transformed host cells for expressing it, pharmaceutical compositions containing it, and uses thereof in medical and diagnostic applications.

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RECOMBINANT VASCULAR ENDOTHELIAL CELL GROWTH FACTOR D (VEGF-D)

This invention relates to growth factors for endothelial cells, and in particular to a novel vascular endothelial growth factor, DNA encoding the factor, and to pharmaceutical and diagnostic compositions and methods utilising or derived from the factor.

BACKGROUND OF THE INVENTION

Angiogenesis is a fundamental process required for normal growth and development of tissues, and involves the proliferation of new capillaries from pre-existing blood vessels. Angiogenesis is not only involved in embryonic development and normal tissue growth, repair, and regeneration, but is also involved in the female reproductive cycle, establishment and maintenance of pregnancy, and in repair of wounds and fractures. In addition to angiogenesis which takes place in the normal individual, angiogenic events are involved in a number of pathological processes, notably tumour growth and metastasis, and other conditions in which blood vessel proliferation, especially of the microvascular system, is increased, such as diabetic retinopathy, psoriasis and arthropathies. Inhibition of angiogenesis is useful in preventing or alleviating these pathological processes.

On the other hand, promotion of angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral

circulation in tissue infarction or arterial stenosis, such as in coronary heart disease and thromboangitis obliterans.

Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor α (TGFα), and hepatocyte growth factor (HGF). See for example Folkman *et al*, "Angiogenesis", J. Biol. Chem., 1992 267 10931-10934 for a review.

It has been suggested that a particular family of endothelial cell-specific growth factors and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the differentiated cells. These factors are members of the PDGF family, and appear to act via endothelial receptor tyrosine kinases (RTKs). Hitherto four vascular endothelial growth factor subtypes have been identified. Vascular endothelial growth factor (VEGF), now known as VEGF-A, has been isolated from several sources. VEGF-A shows highly specific mitogenic activity against endothelial cells, and can stimulate the whole sequence of events leading to angiogenesis. In addition, it has strong chemoattractant activity towards monocytes, can induce plasminogen activator and plasminogen activator inhibitor in endothelial cells, and can also influence microvascular permeability. Because of the latter activity, it is also sometimes referred to as vascular permeability factor (VPF). The isolation and properties of VEGF have been reviewed; see Ferrara *et al*, "The Vascular Endothelial Growth Factor Family of Polypeptides", J. Cellular Biochem., 1991 47 211-218 and Connolly, "Vascular Permeability Factor: A Unique Regulator of Blood Vessel Function", J. Cellular Biochem., 1991 47 219-223.

More recently, three further members of the VEGF family have been identified. These are designated VEGF-B, described in International Patent Application No. PCT/US96/02957 (WO 96/26736) by Ludwig Institute for Cancer Research and The University of Helsinki, VEGF-C, described in Joukov *et al*, The EMBO Journal, 1996 15 290-298, and VEGF2, described in International Patent Application No. PCT/US94/05291 (WO 95/24473) by Human Genome Sciences, Inc. VEGF-B has closely similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique, screening for cellular proteins which might interact with cellular retinoic acid-binding protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02597 and in Olofsson *et al*, Proc. Natl. Acad. Sci., 1996 93 2576-2581.

VEGF-C was isolated from conditioned media of PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for ability of the medium to produce tyrosine phosphorylation of the endothelial cell-specific receptor tyrosine kinase Flt4, using cells transfected to express Flt4. VEGF-C was purified using affinity chromatography with recombinant Flt4, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov *et al*, The EMBO Journal, 1996 15 290-298.

VEGF2 was isolated from a highly tumorgenic, oestrogen-independent human breast cancer cell line. While this molecule is stated to have about 22% homology to PDGF and 30% homology to VEGF, the method of isolation of the gene encoding VEGF2 is unclear, and no characterization of the biological activity is disclosed.

Vascular endothelial growth factors appear to act by binding to receptor tyrosine kinases of the PDGF-receptor family. Five endothelial cell-specific receptor tyrosine kinases have been identified, namely Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4 (VEGFR-3), Tie and Tek/Tie-2. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The essential, specific role in vasculogenesis and angiogenesis of Flt-1, Flk-1, Tie and Tek/Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and placenta growth factor (PlGF). VEGF-C has been shown to be the ligand for Flt4 (VEGFR-3), and also activates VEGFR-2 (Joukov *et al*, 1996). A ligand for Tek/Tie-2 has been described (International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc.); however, the ligand for Tie has not yet been identified.

The receptor Flt-4 is expressed in venous and lymphatic endothelia in the fetus, and predominantly in lymphatic endothelia in the adult (Kaipainen *et al*, Cancer Res, 1994 54 6571-6577; Proc Natl. Acad. Sci. USA, 1995 92 3566-3570). It has been suggested that VEGF-C may have a primary function in lymphatic endothelium, and a secondary function in angiogenesis and permeability regulation which is shared with VEGF (Joukov *et al*, 1996).

We have now isolated human cDNA encoding a novel protein of the vascular endothelial growth factor family. The novel protein, designated VEGF-D, has structural similarities to other members of this family.

SUMMARY OF THE INVENTION

The invention generally provides an isolated novel growth factor which has the ability to stimulate and/or enhance proliferation or differentiation of endothelial cells, isolated DNA sequences encoding the novel growth

factor, and compositions useful for diagnostic and/or therapeutic applications.

According to one aspect, the invention provides an isolated and purified nucleic acid molecule which encodes a novel polypeptide, designated VEGF-D, which is structurally homologous to VEGF, VEGF-B and VEGF-C. In a preferred embodiment, the nucleic acid molecule is a cDNA which comprises the sequence set out in SEQ ID NO. 1, SEQ ID NO. 4, SEQ ID NO. 6 or SEQ ID NO. 7. This aspect of the invention also encompasses DNA molecules of sequence such that they hybridise under stringent conditions with DNA of SEQ ID NO. 1, SEQ ID NO. 4, SEQ ID NO. 6 or SEQ ID NO. 7. Preferably the DNA molecule able to hybridise under stringent conditions encodes the portion of VEGF-D from amino acid residue 93 to amino acid residue 201, optionally operatively linked to a DNA sequence encoding FLAG™ peptide.

Preferably the cDNA comprises the sequence set out in SEQ ID NO. 4, SEQ ID NO. 6 or SEQ ID NO. 7, more preferably that of SEQ ID NO. 4.

According to a second aspect, the invention provides a polypeptide possessing the characteristic amino acid sequence:

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys
(SEQ ID NO. 2),

said polypeptide having the ability to stimulate proliferation of endothelial cells, and said polypeptide comprising a sequence of amino acids substantially corresponding to the amino acid sequence set out in SEQ ID NO. 3, or a fragment or analogue thereof which has the ability to stimulate one or more of endothelial cell proliferation, differentiation, migration or survival.

These abilities are referred to herein as "biological activities of VEGF-D" and can readily be tested by methods known in the art. Preferably the polypeptide has the

ability to stimulate endothelial cell proliferation or differentiation, including, but not limited to, proliferation or differentiation of vascular endothelial cells and/or lymphatic endothelial cells.

More preferably the polypeptide has the sequence set out in SEQ ID NO. 5, SEQ ID NO. 8 or SEQ ID NO. 9, and most preferably has the sequence set out in SEQ ID NO. 5.

A preferred fragment of the polypeptide invention is the portion of VEGF-D from amino acid residue 93 to amino acid residue 201, optionally linked to FLAG™ peptide. Where the fragment is linked to FLAG™, the fragment is VEGFDΔNΔC, as hereindefined.

Thus polypeptides comprising conservative substitutions, insertions, or deletions, but which still retain the biological activity of VEGF-D, are clearly to be understood to be within the scope of the invention. The person skilled in the art will be well aware of methods which can readily be used to generate such polypeptides, for example the use of site-directed mutagenesis, or specific enzymic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-naturally occurring amino acid or an amino acid analogue may retain the required aspects of the biological activity of VEGF-D. Such compounds can readily be made and tested by methods known in the art, and are also within the scope of the invention.

In addition, variant forms of the VEGF-D polypeptide which result from alternative splicing, as are known to occur with VEGF, and naturally-occurring allelic variants of the nucleic acid sequence encoding VEGF-D are encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides, but which do not

result in any substantial functional alteration of the encoded polypeptide.

As used herein, the term "VEGF-D" collectively refers to the polypeptides of SEQ ID NO. 3, SEQ ID NO. 5, SEQ ID NO. 8 and SEQ ID NO. 9 and fragments or analogues thereof which have the biological activity of VEGF-D as herein defined.

Such variant forms of VEGF-D can be prepared by targeting non-essential regions of the VEGF-D polypeptide for modification. These non-essential regions are expected to fall outside the strongly-conserved regions indicated in the figures herein, especially Figure 2 and Figure 10. In particular, the growth factors of the PDGF family, including VEGF, are dimeric, and VEGF-B, VEGF-C, PlGF, PDGF-A and PDGF-B show complete conservation of 8 cysteine residues in the N-terminal domains, *ie.* the PDGF-like domains (Olofsson *et al*, 1996; Joukov *et al*, 1996). These cysteines are thought to be involved in intra- and inter-molecular disulphide bonding. In addition there are further strongly, but not completely, conserved cysteine residues in the C-terminal domains. Loops 1, 2 and 3 of each subunit, which are formed by intra-molecular disulphide bonding, are involved in binding to the receptors for the PDGF/VEGF family of growth factors (Andersson *et al*: Growth Factors, 1995 12 159-164) As shown herein, the cysteines conserved in previously known members of the VEGF family are also conserved in VEGF-D.

The person skilled in the art thus is well aware that these cysteine residues should be preserved in any proposed variant form, and that the active sites present in loops 1, 2 and 3 also should be preserved. However, other regions of the molecule can be expected to be of lesser importance for biological function, and therefore offer suitable targets for modification. Modified polypeptides can readily be tested for their ability to show the biological activity of

VEGF-D by routine activity assay procedures such as cell proliferation tests.

It is contemplated that some modified VEGF-D polypeptides will have the ability to bind to endothelial cells, *ie.* to VEGF-D receptors, but will be unable to stimulate endothelial cell proliferation, differentiation, migration or survival. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of VEGF-D, and to be useful in situations where prevention or reduction of VEGF-D action is desirable. Thus such receptor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing or non-survival promoting variants of VEGF-D are also within the scope of the invention, and are referred to herein as "receptor-binding but otherwise inactive variants".

According to a third aspect, the invention provides a purified and isolated nucleic acid encoding a polypeptide or polypeptide fragment of the invention. The nucleic acid may be DNA, genomic DNA, cDNA or RNA, and may be single-stranded or double stranded. The nucleic acid may be isolated from a cell or tissue source, or of recombinant or synthetic origin. Because of the degeneracy of the genetic code, the person skilled in the art will appreciate that many such coding sequences are possible, where each sequence encodes the amino acid sequence shown in SEQ ID NO. 3, SEQ ID NO. 5, SEQ ID NO. 8 or SEQ ID NO. 9, an active fragment or analogue thereof, or a receptor-binding but otherwise inactive or partially inactive variant thereof.

A fourth aspect of the invention provides vectors comprising the cDNA of the invention or a nucleic acid according to the third aspect of the invention, and host cells transformed or transfected with nucleic acids or vectors of the invention. These cells are particularly suitable for expression of the polypeptide of the invention, and include insect cells such as Sf9 cells, obtainable from the American Type Culture Collection (ATCC SRL-171),

transformed with a baculovirus vector, and the human embryo kidney cell line 293EBNA transfected by a suitable expression plasmid. Preferred vectors of the invention are expression vectors in which a nucleic acid according to the invention is operatively connected to one or more appropriate promoters and/or other control sequences, such that appropriate host cells transformed or transfected with the vectors are capable of expressing the polypeptide of the invention. Other preferred vectors are those suitable for transfection of mammalian cells, or for gene therapy, such as adenovirus or retrovirus vectors or liposomes. A variety of such vectors is known in the art.

The invention also provides a method of making a vector capable of expressing a polypeptide encoded by a nucleic acid according to the invention, comprising the steps of operatively connecting the nucleic acid to one or more appropriate promoters and/or other control sequences, as described above.

The invention further provides a method of making a polypeptide according to the invention, comprising the steps of expressing a nucleic acid or vector of the invention in a host cell, and isolating the polypeptide from the host cell or from the host cell's growth medium. In one preferred embodiment of this aspect of the invention, the expression vector further comprises a sequence encoding an affinity tag, such as FLAG™ or hexahistidine, in order to facilitate purification of the polypeptide by affinity chromatography.

In yet a further aspect, the invention provides an antibody specifically reactive with a polypeptide of the invention. This aspect of the invention includes antibodies specific for the variant forms, fragments and analogues of VEGF-D referred to above. Such antibodies are useful as inhibitors or agonists of VEGF-D and as diagnostic agents for detection and quantification of VEGF-D. Polyclonal or monoclonal antibodies may be used. Monoclonal and

polyclonal antibodies can be raised against polypeptides of the invention using standard methods in the art. For some purposes, for example where a monoclonal antibody is to be used to inhibit effects of VEGF-D in a clinical situation, it may be desirable to use humanized or chimeric monoclonal antibodies. Methods for producing these, including recombinant DNA methods, are also well known in the art.

This aspect of the invention also includes an antibody which recognises VEGF-D and which is suitably labelled.

Polypeptides or antibodies according to the invention may be labelled with a detectable label, and utilised for diagnostic purposes. Similarly, the thus-labelled polypeptide of the invention may be used to identify its corresponding receptor *in situ*. The polypeptide or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels, the latter including enzyme labels or labels of the biotin/avidin system, may be used.

Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in wound healing, tissue or organ transplantation, or to establish collateral circulation in tissue infarction or arterial stenosis, such as coronary artery disease, and inhibition of angiogenesis in the treatment of cancer or of diabetic retinopathy. Quantitation of VEGF-D in cancer biopsy specimens may be useful as an indicator of future metastatic risk.

Inasmuch as VEGF-D is highly expressed in the lung, and it also increases vascular permeability, it is relevant to a variety of lung conditions. VEGF-D assays could be used in the diagnosis of various lung disorders. VEGF-D could also be used in the treatment of lung disorders to improve blood circulation in the lung and/or gaseous exchange between the lungs and the blood stream. Similarly, VEGF-D

could be used to improve blood circulation to the heart and O_2 gas permeability in cases of cardiac insufficiency. In like manner, VEGF-D could be used to improve blood flow and gaseous exhange in chronic obstructive airway disease.

Conversely, VEGF-D antagonists (e.g. antibodies and/or inhibitors) could be used to treat in conditions, such as congestive heart failure, involving accumulations of fluid in, for example, the lung resulting from increases in vascular permeability, by exerting an offsetting effect on vascular permeability in order to counteract the fluid accumulation.

VEGF-D is also expressed in the small intestine and colon, and administrations of VEGF-D could be used to treat malabsorptive syndromes in the intestinal tract as a result of its blood circulation increasing and vascular permeabiltiy increasing activities.

Thus the invention provides a method of stimulation of angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective dose of VEGF-D, or a fragment or analogue thereof which has the ability to stimulate endothelial cell proliferation, to the mammal.

Optionally VEGF-D may be administered together with, or in conjunction with, one or more of VEGF-A, VEGF-B, VEGF-C, PlGF, PDGF, FGF and/or heparin.

Conversely the invention provides a method of inhibiting angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective amount of an antagonist of VEGF-D to the mammal. The antagonist may be any agent that prevents the action of VEGF-D, either by preventing the binding of VEGF-D to its corresponding receptor or the target cell, or by preventing activation of the transducer of the signal from the receptor to its cellular site of action. Suitable antagonists include, but are not limited to, antibodies directed against VEGF-D; competitive or non-

competitive inhibitors of binding of VEGF-D to the VEGF-D receptor, such as the receptor-binding but non-mitogenic VEGF-D variants referred to above; and anti-sense nucleotide sequences complementary to at least a part of the DNA sequence encoding VEGF-D.

The invention also provides a method of detecting VEGF-D in a biological sample, comprising the step of contacting the sample with a reagent capable of binding VEGF-D, and detecting the binding. Preferably the reagent capable of binding VEGF-D is an antibody directed against VEGF-D, more preferably a monoclonal antibody. In a preferred embodiment the binding and/or extent of binding is detected by means of a detectable label; suitable labels are discussed above.

Where VEGF-D or an antagonist is to be used for therapeutic purposes, the dose and route of application will depend upon the condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include subcutaneous, intramuscular or intravenous injection, topical application, implants etc. Topical application of VEGF-D may be used in a manner analogous to VEGF.

According to yet a further aspect, the invention provides diagnostic/prognostic means typically in the form of test kits. For example, in one embodiment of the invention there is provided a diagnostic/prognostic test kit comprising an antibody to VEGF-D and means for detecting, and more preferably evaluating, binding between the antibody and VEGF-D. In one preferred embodiment of the diagnostic/prognostic means according to the invention, either the antibody or the VEGF-D is labelled with a detectable label, and either the antibody or the VEGF-D is substrate-bound, such that the VEGF-D-antibody interaction can be established by determining the amount of label attached to the substrate following binding between the antibody and the VEGF-D. In a particularly preferred

embodiment of the invention, the diagnostic/prognostic means may be provided as a conventional ELISA kit.

In another alternative embodiment, the diagnostic/prognostic means may comprise polymerase chain reaction means for establishing the genomic sequence structure of a VEGF-D gene of a test individual and comparing this sequence structure with that disclosed in this application in order to detect any abnormalities, with a view to establishing whether any aberrations in VEGF-D expression are related to a given disease condition.

In accordance with a further aspect, the invention relates to a method of detecting aberrations in VEGF-D gene structure in a test subject which may be associated with a disease condition in said test subject. This method comprises providing a DNA sample from said test subject; contacting the DNA sample with a set of primers specific to VEGF-D DNA operatively coupled to a polymerase and selectively amplifying VEGF-D DNA from the sample by polymerase chain reaction, and comparing the nucleotide sequence of the amplified VEGF-D DNA from the sample with the nucleotide sequences set forth in SEQ ID NO:1 or SEQ ID NO:4. The invention also includes the provision of a test kit comprising a pair of primers specific to VEGF-D DNA operatively coupled to a polymerase, whereby said polymerase is enabled to selectively amplify VEGF-D DNA from a DNA sample.

Another aspect of the invention concerns the provision of a pharmaceutical composition comprising either VEGF-D polypeptide or a fragment or analogue thereof which promotes proliferation of endothelial cells, or an antibody thereto. Compositions which comprise VEGF-D polypeptide may optionally further comprise one or more of VEGF, VEGF-B and VEGF-C, and/or heparin.

In another aspect, the invention relates to a protein dimer comprising VEGF-D polypeptide, particularly a disulphide-linked dimer. The protein dimers of the

invention include both homodimers of VEGF-D polypeptide and heterodimers of VEGF-D and VEGF, VEGF-B, VEGF-C, PlGF or PDGF.

According to a yet further aspect of the invention there is provided a method for isolation of VEGF-D comprising the step of exposing a cell which expresses VEGF-D to heparin to facilitate release of VEGF-D from the cell, and purifying the thus-released VEGF-D.

Another aspect of the invention involves providing a vector comprising an anti-sense nucleotide sequence which is complementary to at least a part of a DNA sequence which encodes VEGF-D or a fragment or analogue thereof which promotes proliferation of endothelial cells. According to a yet further aspect of the invention such a vector comprising an anti-sense sequence may be used to inhibit, or at least mitigate, VEGF-D expression. The use of a vector of this type to inhibit VEGF-D expression is favoured in instances where VEGF-D expression is associated with a disease, for example where tumours produce VEGF-D in order to provide for angiogenesis. Transformation of such tumour cells with a vector containing an anti-sense nucleotide sequence would suppress or retard angiogenesis, and so would inhibit or retard growth of the tumour.

Polynucleotides of the invention such as those described above, fragments of those polynucleotides, and variants of those polynucleotides with sufficient similarity to the non-coding strand of those polynucleotides to hybridise thereto under stringent conditions all are useful for identifying, purifying, and isolating polynucleotides encoding other, non-human, mammalian forms of VEGF-D. Thus, such polynucleotide fragments and variants are intended as aspects of the invention. Exemplary stringent hybridisation conditions are as follows: hybridisation at 42°C in 5X SSC, 20 mM $NaPO_4$, pH 6.8, 50% formamide; and washing at 42°C in 0.2X SSC. Those skilled in the art understand that it is desirable to vary these conditions empirically based on the

length and the GC nucleotide base content of the sequences to be hybridised, and that formulae for determining such variation exist. See for example Sambrook *et al*, "Molecular Cloning: A Laboratory Manual", Second Edition, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).

Moreover, purified and isolated polynucleotides encoding other, non-human, mammalian VEGF-D forms also are aspects of the invention, as are the polypeptides encoded thereby, and antibodies that are specifically immunoreactive with the non-human VEGF-D variants. Thus, the invention includes a purified and isolated mammalian VEGF-D polypeptide, and also a purified and isolated polynucleotide encoding such a polypeptide.

It will be clearly understood that nucleic acids and polypeptides of the invention may be prepared by synthetic means or by recombinant means, or may be purified from natural sources.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a comparison between the sequences of human VEGF-D and human $VEGF_{165}$ (Figure 1a), human VEGF-B (Figure 1b), human VEGF-C (Figure 1c) and human PlGF (Figure 1d). The box indicates residues which match those in human VEGF-D exactly;

Figure 2 shows sequence alignments between the sequences of human VEGF-D, human $VEGF_{165}$, human VEGF-B, human VEGF-C and human PlGF. The boxes indicate residues that match the VEGF-D sequence exactly; and

Figure 3 shows the amino acid sequence of human VEGF-D (SEQ ID NO 3), as predicted from the cDNA sequence (SEQ ID NO 1). The boxes indicate potential sites for N-linked glycosylation.

Figure 4 shows the nucleotide sequence of a second cDNA sequence encoding human VEGF-D (SEQ ID NO 4), isolated by hybridisation from a commercial human lung cDNA library; this cDNA contains the entire coding region for human VEGF-D;

Figure 5 shows the amino acid sequence for human VEGF-D (SEQ ID NO 5) deduced from the sequence of the cDNA of Figure 4;

Figure 6 shows the nucleotide sequence of cDNA encoding mouse VEGF-D1 (SEQ ID NO 6), isolated by hybridisation screening for a commercially-available mouse lung cDNA library;

Figure 7 shows the nucleotide sequence of cDNA encoding mouse VEGF-D2 (SEQ ID NO 7), isolated from the same library as in Figure 6;

Figure 8 shows the deduced amino acid sequences for mouse VEGF-D1 (SEQ ID NO 8) and VEGF-D2 (SEQ ID NO 9);

Figure 9 shows a comparison between the deduced amino acid sequences of mouse VEGF-D1, mouse VEGF-D2 and human VEGF-D;

Figure 10 shows sequence alignments between the amino acid sequences of human VEGF-D, human $VEGF_{165}$, human VEGF-B, human VEGF-C and human PlGF; and

Figure 11 shows the results of a bioassay in which conditioned medium from COS cells expressing either VEGF-A or VEGF-D was tested for ability to bind to the extracellular domain of a chimeric receptor expressed in Ba/F3 cells.

Figure 12 shows the results of immunoprecipitation and Western blotting analysis of VEGF-D peptides

(A) pEFBOSVEGFD*full*FLAG and pCDNA-1VEGF-A were transfected into COS cells and biosynthetically labelled with ^{35}S-cysteine/methionine for 4 hours. The supernatants from these cultures were immunoprecipitated with either M2 gel or an antiserum directed to VEGF-A coupled to protein A. Washed beads were eluted with an equal volume of 2 x SDS-PAGE sample buffer and boiled. The samples were then resolved by 12% SDS-PAGE. Lanes marked with an asterix (*) indicate where samples were reduced with dithiothreitol and alkylated with iodoacetamide. Molecular weight markers are indicated. *f*A and *f*B indicate the 43 kD and 25 kD species

immunoprecipitated by the M2 gel from the COS cells expressing pEFBOSVEGFD*full*FLAG.

(B) Western blotting analysis of purified VEGFDΔNΔC. An aliquot of material eluted from the M2 affinity column (fraction #3, VEGFDΔNΔC) was combined with 2 x SDS-PAGE sample buffer and resolved on a 15% SDS-PAGE gel. The proteins were then transferred to nitrocellulose membrane and probed with either monoclonal antibody M2 or a control isotype-matched antibody (Neg). Blots were developed using a goat anti-mouse-HRP secondary antibody and chemiluminescence (ECL , Amersham). Monomeric VEGFDΔNΔC is arrowed, as is the putative dimeric form of this peptide (VEGFDΔNΔC"). Molecular weight markers are indicated.

Figure 13 shows the results of analysis of VEGFDΔNΔC protein using the VEGFR2 bioassay. Recombinant VEGFDΔNΔC, and material purified by M2 affinity chromatography, was assessed using the VEGFR2 bioassay. Bioassay cells (10^4), washed to remove IL-3, were incubated with aliquots of conditioned medium from VEGF-D transfected COS cells, fraction #1 from the affinity column (void volume) or fraction #3 from the affinity column (containing VEGFDΔNΔC). All samples were tested at an initial concentration of 20% (ie 1/5) followed by doubling dilutions. Cells were allowed to incubate for 48 hours at 37°C in a humidified atmosphere of 10% CO_2. Cell proliferation was quantitated by the addition of 1 μCi of ^{3}H-thymidine and counting the amount incorporated over a period of 4 hours.

Figure 14 shows stimulation of tyrosine phosphorylation of the VEGFR3 receptor (Flt4) on NIH3T3 cells by culture supernatant from HF cells infected with a recombinant baculovirus vector transformed with VEGF-D.

Figure 15 shows stimulation of tyrosine phosphorylation of the VEGFR2 receptor (KDR) in PAE cells by culture supernatant prepared as in Figure 14.

Figure 16 shows the mitogenic effect of VEGFDΔNΔC on bovine aortic endothelial cells (BAEs). BAEs were treated

with fraction #3 containing VEGFDΔNΔC and, as positive control, purified VEGF-A as described in the text. The result obtained using medium without added growth factor is denoted Medium Control.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by reference to the figures, and to the following non-limiting examples.

Example 1

It has been speculated that no further members of the VEGF family will be found, because there are no known orphan receptors in the VEGFR family. Furthermore, we are not aware of any suggestion in the prior art that other such family members would exist.

A computer search of nucleic acid databases was carried out incidentally to another project, using as search topics the amino acid sequences of VEGF, VEGF-B, VEGF-C and PlGF. Several cDNA sequences were identified by this search. One of these sequences, GenBank Accession No. H24828, encoded a polypeptide which was similar in structure to the cysteine-riched C-terminal region of VEGF-C. This sequence was obtained from the database of expressed sequence tags (dbEST), and for the purposes of this specification is designated XPT. The XPT cDNA had been isolated from a human cDNA library designated "Soares Breast 3NbHBst", which was constructed using mRNA from an adult human female breast tissue. As far as can be ascertained this was normal breast tissue. Sequencing of the XPT DNA was performed pursuant to the Integrated Molecular Analysis of Genome Expression Consortium (IMAGE Consortium), which solicits cDNA libraries from laboratories around the world, arrays the cDNA clones, and provides them to other organisations for sequencing.

The XPT sequence shown in the database was 419 nucleotides long, and encoded an amino acid sequence similar

to the C-terminal 100 amino acids of VEGF-C, *ie.* approximately residues 250 to 350, using the numbering system of Joukov *et al* (1996). Similarly cysteine-rich regions are found in other proteins, which are entirely unrelated in function to the VEGF family, for example the secreted silk-like protein sp185 synthesized in the salivary glands of the midge *Chironomus tentans*. This protein is encoded by the gene BR3, located in a Balbiani ring, a tissue specific chromosome "puff" found on polytene chromosomes in the midge salivary gland (Dignam and Case: Gene, 1990 88 133-140; Paulsson *et al*, J. Mol. Biol., 1990 211 331-349). It is stated in Joukov *et al* (1996) that the sp185-like structural motif in VEGF-C may fold into an independent domain, which is thought to be at least partially cleaved off after biosynthesis, and that there is at least one cysteine motif of the sp185 type in the C-terminal region of VEGF.

Figure 3 of Joukov *et al* shows that the last two-thirds of the C-terminal cysteine-rich region of VEGF-C do not align with VEGF or PlGF, and in fact could be considered a C-terminal extension of VEGF-C which is not present in VEGF or PlGF. The sequence encoded by XPT is similar to this extension. As the XPT cDNA was truncated at its 5' end, it was not possible to deduce or predict any amino acid sequence for regions N-terminal to the cysteine-rich domain. Thus the portion of VEGF-C which is similar to the XPT-derived sequence does not extend to regions of VEGF-C which are conserved among other members of the VEGF family.

As described above, it was not possible to predict whether the N-terminal region of the polypeptide encoded by a full-length XPT nucleic acid (as distinct from the truncated XPT cDNA reported in dbEST) would show any further homology to any member of the VEGF family, in particular VEGF-C, which has a further N-terminal 250 amino acids. For example, the naturally-occurring protein encoded by a full-length XPT nucleic acid could have been the human homologue

of the midge salivary gland protein. Alternatively, the type of cysteine-rich motif encoded by truncated XPT cDNA could be widely distributed among proteins, as are many structural domains. For example, clusters of cysteine residues may be involved in metal binding, formation of intramolecular disulphide bonds to promote accurate protein folding, or formation of intermolecular disulphide bonds for assembly of protein subunits into complexes (Dignam and Chase, 1990). In order to determine whether the truncated XPT cDNA was derived from sequences encoding a VEGF-related molecule, it was necessary to isolate a much longer cDNA.

Example 2 Cloning of cDNA Encoding VEGF-D

A sample of the XPT cDNA reported in dbEST was obtained from the American Type Culture Collection, which is a registered supplier of cDNA clones obtained by the IMAGE Consortium. The identity of the XPT cDNA was confirmed by nucleotide sequencing, using the dideoxy chain termination method (Sanger *et al*, Proc. Natl. Acad. Sci. USA, 1977 74 5463-5467).

The XPT cDNA was used as a hybridisation probe to screen a human breast cDNA library, which was obtained commercially from Clontech. One positive clone was isolated, and this clone was then sequenced on both strands. The nucleotide sequence was compiled, and an open reading frame was identified. The nucleic acid sequence is set out in SEQ ID NO. 1. The polypeptide encoded by this sequence was designated VEGF-D, and its deduced amino acid sequence, designated SEQ ID NO. 3, is set out in Figure 3. In Figure 3 putative sites of N-linked glycosylation, with the consensus sequence N-X-S/T in which X is any amino acid, are indicated by the boxes.

Example 3 Characteristics of VEGF-D

The amino acid sequence of VEGF-D was compared with those of human VEGF-A_{165}, VEGF-B, VEGF-C and PlGF. These

comparisons are set out in Figures 1a to d respectively. The degree of sequence homology was calculated, and if gaps in sequence introduced for the purposes of alignment are not considered in the calculation, VEGF-D is 31% identical to VEGF, 48% identical to VEGF-C, 28% identical to VEGF-B and 32% identical to PlGF. Thus the most closely-related protein identified was VEGF-C.

Computer searches of the GenBank, EMBL and SwissProt nucleic acid databases did not reveal any protein sequences identical to VEGF-D. As expected from the sequence alignment referred to above, the most closely related protein found in these databases was VEGF-C. Searches of dbEST were also performed, but did not reveal any sequences encompassing the entire coding region of VEGF-D. The sequence of VEGF-D is unrelated to that of Tie-2 ligand 1 as disclosed in WO 96/11269.

It is important to bear in mind that the only homologies detected were at the level of amino acid sequence. Thus it would not have been possible to isolate the cDNA or gDNA encoding VEGF-D by methods such as low-stringency hybridization with a nucleic acid sequence encoding another member of the VEGF family.

VEGF-D appears to be most closely related to VEGF-C of all the members of the VEGF family. Because the VEGF-D amino acid sequence includes the cysteine-rich sp185-like motif which is found in VEGF-C, the polypeptide of the invention may play an important functional role in lymphatic endothelia. While we do not wish to be bound by any proposed mechanism, it is thought that VEGF-C and VEGF-D may constitute a silk-like matrix over which endothelial cells can grow. Lymphatic vessels have no basement membrane, so the silk-like matrix can form a basement membrane-like material. This may be important in promoting cell growth and/or in cell differentiation, and may be relevant to cancer, especially metastasis, drug therapy, cancer prognosis, etc.

Example 4 Biological Characteristics of VEGF-D

The cDNA sequence of VEGF-D was used to predict the deduced amino acid sequence of VEGF-D, the biochemical characteristics of the encoded polypeptide, including the numbers of strongly basic, strongly acidic, hydrophobic and polar amino acids, the molecular weight, the isoelectric point, the charge at pH 7, and the compositional analysis of the whole protein. This analysis was performed using the Protean protein analysis program, Version 1.20 (DATASTAR). These results are summarised in Tables 1 and 2 below. Table 1 also shows the codon usage.

Table 1

Translated DNA Sequence of VEGF-D contig x(1,978)
With Standard Genetic Code

Molecular Weight 37056.60 Daltons
425 Amino Acids
46 Strong Basic(+) Amino Acids (K,R)
41 Strong Acidic(-) Amino Acids (D,E)
79 hydrophobic Amino Acids (A,I,L,F,W,V)
108 Polar Amino Acids (N,C,Q,S,T,Y)

7.792 Isoelectric Point
6.371 Charge at pH 7.0

Total number of bases translated is 978
% A = 28.73 [281]
% G = 23.11 [226]
% T = 23.21 [227]
% C = 24.95 [244]
% Ambiguous = 0.00 [0]
% A+T = 51.94 [508]
% C+G = 48.06 [470]

Davis, Botstein, Roth Melting Temp °C. 84.09
Wallace Temp °C. 3384.00

Table 1 (cont.)

Codon usage:				
ccg ( )	0 # ugc Cys(C)	14 # cuc Leu(L)	6 # ucg Ser(S)	
uaa ( )	0 # ugu Cys(C)	16 # cug Leu(L)	4 # ucu Ser(S)	
uag ( )	0 # --- Cys(C)	30 # cuu Leu(L)	2 # --- Ser(S)	3
--- ( )	0 # caa Gln(Q)	1 # uua Leu(L)	1 # uga Ter(.)	
gca Ala(A)	5 # cag Gln(Q)	11 # uug Leu(L)	5 # --- Ter(.)	
gcc Ala(A)	4 # --- Gln(Q)	12 # --- Leu(L)	23 # aca Thr(T)	
gcg Ala(A)	1 # gaa Glu(E)	16 # aaa Lys(K)	13 # acc Thr(T)	
gcu Ala(A)	5 # gag Glu(E)	12 # aag Lys(K)	10 # acg Thr(T)	
--- Ala(A)	15 # --- Glu(E)	28 # --- Lys(K)	23 # acu Thr(T)	
aga Arg(R)	7 # gga Gly(G)	1 # aug Met(M)	6 # --- Thr(T)	2
agg Arg(R)	5 # ggc Gly(G)	2 # --- Met(M)	6 # ugg Trp(W)	
cga Arg(R)	5 # ggg Gly(G)	3 # uuc Phe(F)	4 # --- Trp(W)	
cgc Arg(R)	4 # ggu Gly(G)	2 # uuu Phe(F)	8 # uac Tyr(Y)	
cgg Arg(R)	1 # --- Gly(G)	8 # --- Phe(F)	12 # uau Tyr(Y)	
cgu Arg(R)	1 # cac His(H)	7 # cca Pro(P)	9 # --- Tyr(Y)	
--- Arg(R)	23 # cau His(H)	7 # ccc Pro(P)	6 # gua Val(V)	
aac Asn(N)	5 # --- His(H)	14 # ccu Pro(P)	8 # guc Val(V)	
aau Asn(N)	4 # aua Ile(I)	2 # --- Pro(P)	23 # gug Val(V)	
--- Asn(N)	9 # auc Ile(I)	6 # agc Ser(S)	6 # guu Val(V)	
gac Asp(D)	8 # auu Ile(I)	5 # agu Ser(S)	8 # --- Val(V)	1
gau Asp(D)	5 # --- Ile(I)	13 # uca Ser(S)	5 # nnn ???(X)	
gau Asp(D)	5 # --- Ile(I)	13 # uca Ser(S)	5 # nnn ???(X)	
--- Asp(D)	13 # cua Leu(L)	5 # ucc Ser(S)	7 # TOTAL	32

Contig 2:

Contig Length: 2379 bases

Average Length/Sequence: 354 bases

Total Sequence Length: 4969 bases

Table 2

Predicted Structural Class of the Whole Protein:

Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	**Whole Protein**
Molecular Weight	37056.60 m.w.
Length	325
1 microgram =	26.986 pMoles
Molar Extinction coefficient	30200±5%
1 A(280) =	1.23 mg/ml
Isoelectric Point	7.79
Charge at pH 7	6.37

Table 2 (cont.)

Whole Protein Composition Anayalsis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	134	46.30	41.23
Acidic (DE)	41	13.79	12.62
Basic (KR)	46	17.65	14.15
Polar (NCQSTY)	108	30.08	33.23
Hydrophobic (AILFWV)	79	23.86	24.31
A Ala	15	2.88	4.62
C Cys	30	8.35	9.23
D Asp	13	4.04	4.00
E Glu	28	9.75	8.62
F Phe	12	4.77	3.69
G Gly	8	1.23	2.46
H His	14	5.18	4.31
I Ile	13	3.97	4.00
K Lys	23	7.96	7.08
L Leu	23	7.03	7.08
M Met	6	2.12	1.85
N Asn	9	2.77	2.77
P Pro	23	6.08	7.08
Q Gln	12	4.15	3.69
R Arg	23	9.69	7.08
S Ser	33	7.76	10.15
T Thr	21	5.73	6.46
V Val	12	3.21	3.69
W Trp	4	2.01	1.23
Y Trp	3	1.32	0.92
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

This analysis predicts a molecular weight for the unprocessed VEGF-D monomer of 37 kilodaltons (kD), compared to the experimentally determined values (for the fully processes peptides) of 20 to 27 kD for VEGF-A monomers, 21 kD for the VEGF-B monomer and 23 kD for the VEGF-C monomer.

Example 5

The original isolation of a cDNA for VEGF-D, described in Example 2 involved hybridisation screening of a human breast cDNA library. As only one cDNA clone for VEGF-D was thus isolated, it was not possible to confirm the structure of the cDNA by comparison with other independently isolated VEGF-D cDNAs. The work described in this example, which involved isolation of additional human VEGF-D cDNA clones, was carried out in order to confirm the structure of human VEGF-D cDNA. In addition, mouse VEGF-D cDNA clones were isolated.

Two cDNA libraries which had been obtained commercially from Stratagene, one for human lung and one for mouse lung (catalogue numbers 937210 and 936307, respectively) were used for hybridisation screening with a VEGF-D cDNA probe. The probe, which spanned from nucleotides 1817 to 2495 of the cDNA for human VEGF-D described in Example 2, was generated by polymerase chain reaction (PCR) using a plasmid containing the VEGF-D cDNA as template and the following two oligonucleotides:

5'-GGGCTGCTTCTAGTTTGGAG (SEQ ID NO. 10), and

5'-CACTCGCAACGATCTTCGTC (SEQ ID NO. 11).

Approximately two million recombinant bacteriophage were screened with this probe from each of the two cDNA libraries. Nine human and six mouse cDNA clones for VEGF-D were subsequently isolated.

Two of the nine human cDNA clones for VEGF-D were sequenced completely using the dideoxy chain termination method (Sanger *et al*, Proc. Natl. Acad. Sci. USA, 1977 74

5463-5467). The two cDNAs contained the entire coding region for human VEGF-D, and were identical except that one of the clones was five nucleotides longer than the other at the 5'-terminus. The nucleotide sequence of the shorter cDNA is shown in Figure 4, and is designated SEQ ID NO. 4. The amino acid sequence for human VEGF-D (hVEGF-D) deduced from this cDNA was 354 residues long, and is shown in Figure 5; this is designated SEQ ID NO. 5. The sequences of the 5' regions of five of the other human VEGF-D cDNA clones were also determined. For each clone, the sequence that was characterized contained more than 100 nucleotides of DNA immediately downstream from the translation start site of the coding region. In all cases, the sequences of these regions were identical to corresponding regions of the human VEGF-D cDNA shown in Figure 4.

All six mouse cDNA clones for VEGF-D were sequenced completely. Only two of the clones contained an entire coding region for VEGF-D; the other clones were truncated. The nucleotide sequences of the two clones with an entire coding region are different, and encode amino acid sequences of different sizes. The longer amino acid sequence is designated mVEGF-D1, and the shorter sequence is designated mVEGF-D2. The nucleotide sequences of the cDNAs encoding mVEGF-D1 and mVEGF-D2 are shown in Figures 6 and 7 respectively. The deduced amino acid sequences for mVEGF-D1 and mVEGF-D2 are shown in Figure 8. These sequences are respectively designated SEQ ID NOS. 6, 7, 8 and 9. The differences between the amino acid sequences are:

i) an insertion of five amino acids (DFSFE) after residue 30 in mVEGF-D1 in comparison to mVEGF-D2;

ii) complete divergence of the C-terminal ends after residue 317 in mVEGF-D1 and residue 312 in mVEGF-D2, which results in mVEGF-D1 being considerably longer.

Three of the four truncated cDNAs for mouse VEGF-D encoded the C-terminal region, but not the N-terminal 50 amino acids. All three of these cDNAs encoded a C-terminal end for VEGF-D which is identical to that for mVEGF-D2. The other truncated cDNA encoded only the N-terminal half of VEGF-D. The amino acid sequence deduced from this cDNA contained the five amino acids DFSFE immediately after residue 30 found in mVEGF-D1, but not in mVEGF-D2.

As described above, the entire sequence of the human VEGF-D cDNA clone reported in this example has been validated by comparison with that for a second human clone. In addition, the sequence of the 5' end of the coding region was found to be identical in five other human VEGF-D cDNA clones. In contrast, the sequence reported in Example 2 contained most of the coding region for VEGF-D, but was incorrect near the 5'-end of this region. This was probably because the VEGF-D cDNA was truncated near the 5'-end of the coding region and at that point had been ligated with another unidentified cDNA, and consequently the first 30 codons of the true coding sequence for VEGF-D had been deleted and replaced with a methionine residue. This methionine residue was defined as the N-terminal amino acid of the VEGF-D sequence presented in Example 2.

The N-terminal regions of the deduced amino acid sequences of mouse VEGF-D1 and VEGF-D2 are very similar to that deduced for human VEGF-D (see Figure 9). This also indicates that the correct deduced amino acid sequence for human VEGF-D is reported in this example. The N-terminal 25 amino acids of human VEGF-D form an extremely hydrophobic region, which is consistent with the notion that part of this region may be a signal sequence for protein secretion. Figure 10 shows the alignment of the human VEGF-D sequence with the sequences of other members of the VEGF family of growth factors, namely human $VEGF_{165}$

($hVEGF_{165}$), human VEGF-B (hVEGF-B), human VEGF-C (hVEGF-C) and human Placental Growth Factor (hPlGF). When gaps in the alignments are ignored for the purposes of calculation, human VEGF-D is found to be 31% identical in amino acid sequence to human $VEGF_{165}$, 28% identical to human VEGF-B, 48% identical to VEGF-C and 32% identical to human PlGF. Clearly VEGF-C is the member of this family which is most closely related to VEGF-D.

The differences in sequence for mouse VEGF-D1 and VEGF-D2 most probably arise from differential mRNA splicing. The C-terminal 41 amino acid residues of VEGF-D1 are deleted in VEGF-D2, and are replaced with 9 residues which are not closely related to the VEGF-D1 sequence. Therefore 4 cysteine residues present near the C-terminus of VEGF-D1 are deleted in VEGF-D2. This change may alter the tertiary or quaternary structures of the protein, or may affect the localisation of the protein in the cell or the extracellular environment. The C-terminal end of human VEGF-D resembles that of mouse VEGF-D1, not mouse VEGF-D2. The small 5 amino acid insertion after residue 30 in mouse VEGF-D1, which is not present in either mouse VEGF-D2 or human VEGF-D, may influence proteolytic processing of the protein.

VEGF-D is highly conserved between mouse and man. Eighty-five percent of the amino acid residues of human VEGF-D are identical in mouse VEGF-D1. This is likely to reflect conservation of protein function. Putative functions for VEGF-D have been proposed herein. Although we have not found alternative forms of human VEGF-D cDNA, it is possible that the RNA splice variation which gives rise to numerous forms of mRNA for mouse VEGF-D may also occur in human tissues.

Example 6 Expression of VEGF-D in COS Cells

A fragment of the human cDNA for VEGF-D, spanning from nucleotide 1 to 1520 of the sequence shown in Figure

4 and containing the entire coding region, was inserted into the mammalian expression vector pCDNA1-amp. The vector was used to transiently transfect COS cells by the DEAE-Dextran method as described previously (Aruffo and Seed, 1987) and the resulting conditioned cell culture media, collected after 7 days of incubation, were concentrated using Amicon concentrators (Centricon 10 with a 10,000 molecular weight cut off) according to the manufacturer. The plasmids used for transfections were the expression construct for human VEGF-D and, as positive control, a construct made by insertion of mouse VEGF-A cDNA into pCDNA1-amp. The conditioned media were tested in two different bioassays, as described below, and the results demonstrate that the COS cells did in fact express and secrete biologically-active VEGF-D.

Example 7 Bioassay for Capacity of VEGF-D to Bind to VEGF Receptor-2

As shown in Example 5, VEGF-D is closely related in primary structure to other members of the VEGF family. Most members of this protein family are mitogenic and/or chemotactic for endothelial cells (Keck *et al*, 1989; Leung *et al*, 1989; Joukov, *et al*, 1996; Olofsson *et al*, 1996). In addition VEGF-A (previously known as VEGF), the first member of the VEGF family to be described in the literature, is a potent inducer of vascular permeability (Keck *et al*, 1989). As protein structure is an important determinant of protein function, it seemed likely that VEGF-D might also be mitogenic for endothelial cells or induce vascular permeability. Therefore human VEGF-D was tested in a bioassay for its capacity to bind to VEGF receptor-2 (VEGFR2; also known as Flk-1), an endothelial cell-specific receptor which, when activated by VEGF-A, is thought to give rise to a mitogenic signal (Strawn *et al*, 1996).

A bioassay for detection of growth factors which bind to VEGFR2 has been developed in the factor-dependent cell line Ba/F3, and is described in our earlier patent application, No. PCT/US95/16755. These cells grow in the presence of interleukin-3 (IL-3); however removal of this factor results in cell death within 48 hours. If another receptor capable of delivering a growth stimulus is transfected into the Ba/F3 cells, the cells can be rescued by the specific growth factor which activates that receptor when the cells are grown in medium lacking IL-3. In the specific case of receptor-type tyrosine kinases (eg. VEGFR2), chimeric receptors containing the extracellular domain of the receptor tyrosine kinase and the transmembrane and cytoplasmic domains of the erythropoietin receptor (EpoR) can be utilised. In this case stimulation with the ligand (eg. VEGF), which binds to the extracellular domain of the chimeric receptor, results in signalling via the EpoR cytoplasmic domain and subsequent rescue of the cell line in growth medium lacking IL-3. The construction of the chimeric receptor used in this study, consisting of the mouse VEGFR2 extracellular domain and the mouse EpoR transmembrane and cytoplasmic domains, and the bioassay itself are described below.

Plasmid Construction

i) Construction of a plasmid for generating chimeric VEGFR2 receptors

To obtain a plasmid construct with which DNA encoding the extracellular domain of mouse VEGFR2 could easily be ligated with DNA encoding other protein domains, site-directed mutagenesis was used to generate a BglII restriction enzyme site at the position of mouse VEGFR2 cDNA which encoded the junction of the extracellular domain and the transmembrane domain. The full-length clone of the mouse VEGFR2 cDNA described by Oelrichs *et al*

(1993) was subcloned into the mammalian expression vector pCDNA1-amp, using the BstXI restriction enzyme site. Single stranded UTP+ DNA was generated using the M13 origin of replication, and this was used as a template to generate mouse VEGFR2 cDNA containing the BglII site at the desired position. The plasmid containing the altered VEGFR2 cDNA was designated pVEGFR2Bgl. DNA fragments encoding the transmembrane and cytoplasmic domains of any receptor can be inserted at the BglII site of pVEGFR2Bgl in order to generate chimeric VEGFR2 receptors.

ii) Construction of VEGFR2/EpoR chimeric receptor

The mouse EpoR cDNA was subcloned into the expression vector pCDNA1-amp, and single stranded DNA was generated as a template for mutagenesis. A BglII restriction enzyme site was inserted into the EpoR cDNA at the position encoding the junction of the transmembrane and extracellular domains of the EpoR to allow direct ligation of this DNA fragment to the modified cDNA encoding the extracellular domain of VEGFR2 in pVEGFR2Bgl. In addition a BglII site in the cytoplasmic domain of the EpoR was removed by a silent single nucleotide substitution. The DNA fragment encoding the transmembrane and cytoplasmic domains of EpoR was then used to replace the portion of pVEGFR2Bgl encoding the transmembrane and cytoplasmic domains of VEGFR2. Thus a single reading frame was generated which encoded the chimeric receptor consisting of the VEGFR2 extracellular domain and the EpoR transmembrane and cytoplasmic domains.

The DNA fragment encoding the chimeric receptor was subcloned into the expression vector pBOS, and co-transfected into the Ba/F3 cell line with plasmid pgk-neo at a ratio of 1:20. Cells expressing the VEGFR2-EpoR protein were selected by flow cytometry analysis using a monoclonal antibody to the VEGFR2 extracellular domain (MAb 4H3). This monoclonal antibody is described in

Australian Patent Application No. PM 3794 filed 10 February 1994. Cell lines expressing higher levels of VEGFR2-EpoR were selected by growing the cells in 5 μg/ml MAb 4H3 or 25 ng/ml of recombinant VEGF. A cell line expressing high levels of VEGFR2-EpoR, designated Ba/F3-NYK-EpoR, was used for the bioassay.

The Bioassay

The Ba/F3-NYK-EpoR cells described above were washed three times in PBS to remove all IL-3 and resuspended at a concentration of 1000 cells per 13.5 μl of culture medium and 13.5 μl was aliquoted per well of a 60-well Terasaki plate. Conditioned media from transfected COS cells were then diluted into the cell culture medium. Cells expressing a chimeric receptor consisting of the extracellular domain of the endothelial cell receptor Tie2 and the transmembrane and cytoplasmic domains of EpoR were used as a non-responding control cell line. Cells were incubated for 48-96 hours, during which the cells incubated in cell culture medium alone had died and the relative survival/proliferation seen in the other wells (ie. in the presence of COS cell-conditioned media) was scored by counting the viable cells present per well.

The conditioned medium from COS cells which had been transiently transfected with expression plasmids was concentrated 30-fold and used in the VEGFR2 bioassay. Concentrated conditioned medium from COS cells transfected with pCDNA1-amp was used as negative control.

The results are shown in Figure 11, with the percentage of 30-fold concentrated COS cell-conditioned medium in the incubation medium (vol/vol) plotted versus the number of viable cells in the well after 48 hours of incubation. Clearly the conditioned medium containing either VEGF-A or VEGF-D was capable of promoting cell survival in this assay, indicating that both proteins can bind to and activate VEGFR2.

Example 8 Vascular Permeability Assay

Human VEGF-D, prepared as in Example 6 and concentrated 30-fold, was tested in the Miles vascular permeability assay (Miles and Miles, 1952) performed in anaesthetized guinea pigs (albino/white, 300-400 g). Concentrated conditioned medium for COS cells transfected with pCDNA1-amp was again used as a negative control. Guinea pigs were anaesthetised with chloral-hydrate (3.6 g/100 ml; 0.1 ml per 10 g of body weight). The backs of the animals were then carefully shaved with clippers. Animals were given an intracardiac injection of Evans Blue dye (0.5% in MT PBS, 0.5 ml) using a 23G needle, and were then injected intra-dermally with 100-150 µl of concentrated COS cell-conditioned medium. After 15-20 min the animals were sacrificed and the layer of skin on the back excised to expose the underlying blood vessels. For quantitation, the area of each injection was excised and heated to 45°C in 2-5 ml of formamide. The resulting supernatants, containing extravasated dye, were then examined spectrophotometrically at 620 nm.

For animal 1, the absorbance at 620 nm arising from injection of 30-fold concentrated VEGF-A conditioned medium was 0.178, that for the 30-fold concentrated VEGF-D conditioned medium was 0.114, and that for 30-fold concentrated medium from cells transfected with pCDNA1-amp was 0.004. For animal 2, the 30-fold concentrated media were diluted 4-fold in cell culture medium before intra-dermal injection. The absorbance at 620 nm for the VEGF-A conditioned sample was 0.141, that for the VEGF-D conditioned sample was 0.116 and that for a sample matched for serum content as negative control was 0.017. The enhanced extravasation of dye observed for both animals in the presence of VEGF-A or VEGF-D demonstrated that both of these proteins strongly induced vascular permeability.

The data described here indicate that VEGF-D is a secreted protein which, like VEGF-A, binds to and

activates VEGFR2 and can induce vascular permeability.

Example 9 Bioactivities of Internal VEGF-D Polypeptides

The deduced amino acid sequence for VEGF-D includes a central region which is similar in sequence to all other members of the VEGF family (approximately residues 101 to 196 of the human VEGF-D amino acid sequence as shown in the alignment in Figure 10). Therefore, it was thought that the bioactive portion of VEGF-D might reside in the conserved region. In order to test this hypothesis, the biosynthesis of VEGF-D was studied, and the conserved region of human VEGF-D was expressed in mammalian cells, purified, and tested in bioassays as described below.

Plasmid construction

A DNA fragment encoding the portion of human VEGF-D from residue 93 to 201, *ie.* with N- and C-terminal regions removed, was amplified by polymerase chain reaction with *Pfu* DNA polymerase, using as template a plasmid comprising full-length human VEGF-D cDNA. The amplified DNA fragment, the sequence of which was confirmed by nucleotide sequencing, was then inserted into the expression vector pEFBOSSFLAG to give rise to a plasmid designated pEFBOSVEGFDΔNΔC. The pEFBOSSFLAG vector contains DNA encoding the signal sequence for protein secretion from the interleukin-3 (IL-3) gene and the FLAG™ octapeptide. The FLAG™ octapeptide can be recognized by commercially available antibodies such as the M2 monoclonal antibody (IBI/Kodak). The VEGF-D PCR fragment was inserted into the vector such that the IL-3 signal sequence was immediately upstream from the FLAG™ sequence, which was in turn immediately upstream from the VEGF-D sequence. All three sequences were in the same reading frame, so that translation of mRNA resulting from transfection of pEFBOSVEGFDΔNΔC into mammalian cells would

give rise to a protein which would have the IL-3 signal sequence at its N-terminus, followed by the FLAG™ octapeptide and the VEGF-D sequence. Cleavage of the signal sequence and subsequent secretion of the protein from the cell would give rise to a VEGF-D polypeptide which is tagged with the FLAG™ octapeptide adjacent to the N-terminus. This protein was designated VEGFDΔNΔC.

In addition, a second plasmid was constructed, designated pEFBOSVEGFD*full*FLAG, in which the full-length coding sequence of human VEGF-D was inserted into pEFBOSIFLAG such that the sequence for the FLAG™ octapeptide was immediately downstream from, and in the same reading frame as, the coding sequence of VEGF-D. The plasmid pEFBOSIFLAG lacks the IL-3 signal sequence, so secretion of the VEGF-D/FLAG fusion protein was driven by the signal sequence of VEGF-D. pEFBOSVEGFD*full*FLAG was designed to drive expression in mammalian cells of full-length VEGF-D which was C-terminally tagged with the FLAG™ octapeptide. This protein is designated VEGFD*full*FLAG, and is useful for the study of VEGF-D biosynthesis.

Analysis of the Post-Translational Processing of VEGF-D

To examine whether the VEGF-D polypeptide is processed to give a mature and fully active protein, pEFBOSVEGFD*full*FLAG was transiently transfected into COS cells (Aruffo and Seed, 1987). Expression in COS cells followed by biosynthetic labeling with ^{35}S-methionine/cysteine and immunoprecipitation with M2 gel has demonstrated species of approximately 43 kD (*f*A) and 25 kD(*f*B) (Figure 12A). These bands are consistent with the notion that VEGF-D is cleaved to give a C-terminal fragment (FLAG™ tagged) and an internal peptide (corresponding approximately to the VEGFDΔNΔC protein). Reduction of the immunoprecipitates (M2*) gives some reduction of the *f*A band, indicating the potential for disulphide linkage betwe n the two fragments.

Expression and purification of internal VEGF-D polypeptide

Plasmid pEFBOSVEGFDΔNΔC was used to transiently transfect COS cells by the DEAE-Dextran method as described previously (Aruffo and Seed, 1987). The resulting conditioned cell culture medium (approximately 150 ml), collected after 7 days of incubation, was subjected to affinity chromatography using a resin to which the M2 monoclonal antibody had been coupled. In brief, the medium was run batch-wise over a 1 ml M2 antibody column for approximately 4 hours at 4°C. The column was then washed extensively with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl before elution with free FLAG™ peptide at 25 μg/ml in the same buffer. The resulting material was used for the bioassays described below.

In order to detect the purified VEGFDΔNΔC, fractions eluted from the M2 affinity column were subjected to Western blot analysis. Aliquots of the column fractions were combined with 2 x SDS-PAGE sample buffer, boiled and loaded onto a 15% SDS polyacrylamide gel. The resolved fractions were transferred to nitrocellulose membrane and non-specific binding sites blocked by incubation in Tris/NaCl/Tween 20 (TST) and 10% skim milk powder (BLOTTO). Membranes were then incubated with monoclonal antibody M2 or control antibody at 3 μg/ml for 2 h at room temperature, followed by extensive washing in TST. Membranes were then incubated with a secondary goat anti-mouse HRP-conjugated antiserum for 1 h at room temperature, followed by washing in TST buffer. Detection of the protein species was achieved using a chemiluminescent reagent (ECL, Amersham) (Figure 12B).

Under non-reducing conditions a species of molecular weight approximately 23 kD (VEGFDΔNΔC) was detected by the M2 antibody. This is consistent with the predicted molecular weight for this internal fragment (12,800) plus N-linked glycosylation; VEGFDΔNΔC contains two potential N-linked glycosylation sites. A species of

approximately 40 kD was also detected, and may represent a non-covalent dimer of the 23 kD protein (VEGFDΔNΔC).

Bioassays

The bioassay for the capacity of polypeptides to bind to VEGF receptor-2 is described in detail in Example 7. Aliquots of fractions eluted from the M2 affinity column, containing the VEGFDΔNΔC protein, were diluted in medium and tested in the VEGFR2 bioassay as previously described. Fraction #3 from the affinity column, which was shown to contain the purified VEGFDΔNΔC protein (Figure 12B), demonstrated a clear ability to induce proliferation of the bioassay cell line to a dilution of 1/100 of the purified fraction (Figure 13). In comparison, the void volume of the affinity column (fraction #1) showed no activity, whereas the original VEGFDΔNΔC conditioned medium gave only weak activity.

The vascular permeability assay (Miles and Miles, 1952) is described in brief in example 8. Aliquots of purified VEGFDΔNΔC, and samples of the void volume from the M2 affinity column (negative control) were combined with medium and injected intradermally into the skin of guinea pigs. The regions of skin at the sites of injections were excised, and extravasated dye was eluted. The absorbance of the extravasated dye at 620 nm arising from injection of purified VEGFDΔNΔC was 0.131 ± 0.009. In comparison, the value for absorbance arising from injection of a sample of the void volume was 0.092 ± 0.020. Therefore VEGFDΔNΔC induced vascular permeability, but the effect was only marginal.

Due to its ability to bind to VEGFR2 and its lower induction of vascular permeability compared to full length VEGF-D, VEGF-DΔNΔC may be said to relatively decrease the induction of vascular permeability by VEGF-D through competitive inhibition. In this sense, the VEGF-DΔNΔC fragment may be thought of as an antagonist for VEGF-D as

regards the induction of vascular permeability.

Summary

Two factors have led us to explore internal fragments of VEGF-D for enhanced activity. Firstly, it is the central region of VEGF-D which exhibits amino acid homology with all other members of the VEGF family. Secondly, proteolytic processing which gives rise to internal bioactive polypeptides occurs for other growth factors such as PDGF-BB. In addition, the activity seen with the full length VEGF-D protein in COS cells was lower than for the corresponding conditioned medium from VEGF-A transfected COS cells.

It was predicted that the mature VEGF-D sequence would be derived from a fragment contained within residues 92-205, with cleavage at FAA^TFY and IIRR^SIQI. Immunoprecipitation analysis of VEGF-D*full*FLAG expressed in COS cells produced species consistent with the internal proteolytic cleavage of the VEGF-D polypeptide at these sites. Therefore a truncated form of VEGF-D, with the N- and C-terminal regions removed (VEGFDΔNΔC), was produced and expressed in COS cells. This protein was identified and purified using the M2 antibody. The VEGFDΔNΔC protein was also detected by the A2 antibody, which recognizes a peptide within the 92-205 fragment of VEGF-D (not shown). VEGFDΔNΔC was evaluated by the VEGFR2 bioassay and the Miles vascular permeability assay, and shown to bind to and activate the VEGFR2 receptor in a bioassay designed to detect cross-linking of the VEGFR2 extracellular domain. Induction of vascular permeability by this polypeptide in a Miles assay was at best marginal, in contrast to the effect of VEGF-A.

Example 10 VEGF-D Binds to and Activates VEGFR-3

The human VEGF-D cDNA was cloned into baculovirus shuttle vectors for the production of reconmbinant VEGF-D.

In addition to baculoviral shuttle vectors, which contained the unmodified VEGF-D cDNA (referred to as "full length VEGF-D") two baculoviral shuttle vectors were assembled, in which the VEGF-D cDNA was modified in the following ways.

In one construct (referred to as "full length VEGF-D-H_6") a C-terminal histidine tag was added. In the other construct the putative N- and C-terminal propeptides were removed, the melittin signal peptide was fused in-frame to the N-terminus, and a histidine tag was added to the C-terminus of the remaining VEGF homology domain (referred to as "ΔNΔc-MELsp-VEGF-D-H_6").

For each of the three constructs baculoviral clones of two or three independent transfections were amplified . The supernatant of High Five (HF) cells was harvested 48 h post infection with high titre virus stocks. The supernatant was adjusted to pH 7 with NaOH and diluted with one volume of D-MEM (0.2% FCS).

The samples were tested for their ability to stimulate tyrosine phosphorylation of VEGFR-3 (Flt4 receptor) on NIH3T3 cells, as described by Joukov *et al*, 1996. The supernatant of uninfected cells and the supernatant of cells infected with the short splice variant of VEGF-C, which does not stimulate tyrosine phosphorylation of VEGFR-3, were used as negative controls. VEGF-C modified in the same way as ΔNΔC-melSP-VEGF-D-H_6 was used as positive control. The results are shown in Figure 14.

The appearance of new bands at 125 and 195 kD indicates phosphorylation, and hence activation, of the receptor.

Example 11 VEGF-D Binds to and Activates VEGFR-2

Modified and unmodified human VEGF-D cDNA was cloned into baculovirus shuttle vectors for the production of recombinant VEGF-D as described in Example 10.

For each of the three constructs full length VEGF-D, full length VEGF-D-H_6, and ΔNΔC-melSP-VEGF-D-H_6, baculoviral clones of two or three independent transfections were amplified . The supernatant of High Five (HF) cells was harvested 48 hours post infection with high titre virus stocks. The supernatant was adjusted to pH 7 with NaOH and diluted with one volume of D-MEM (0.2% FCS).

The supernatants conditioned with the histidine-tagged proteins were tested for their ability to stimulate tyrosine phosphorylation of the KDR receptor according to Joukov *et al*, 1996. KDR is the human homologue of flk1 (VEGFR-2).

The supernatant of uninfected cells and the supernatant of cells infected with the VEGF-C 156S mutant, which does not stimulate KDR, were used as negative controls. $VEGF_{165}$ and VEGF-C modified in the same way as ΔNΔC-melSP-VEGF-D-H_6 were used as positive controls. The results are shown in Figure 15.

The appearance of a new band at approximately 210 kD indicates phosphorylation, and hence activation, of the receptor.

Example 12 Analysis of VEGF-D Gene Expression

In order to characterise the pattern of VEGF-D gene expression in the human and in mouse embryos, VEGF-D cDNAs were used as hybridization probes for Northern blot analysis of polyadenylated human RNA and for *in situ* hybridization analysis with mouse embryos.

Gene expression in the adult human

A 1.1 kb fragment of the human VEGF-D cDNA shown in Figure 4 (SEQ ID NO. 4) spanning from the *Eco*RV site to the 3'-terminus (nucleotides 911 to 2029) was labelled with [α-^{32}P]dATP using the Megaprime DNA labelling system (Amersham) according to manufacturer's instructions. This

probe was used to screen human multiple tissue northern blots (Clontech) by hybridization, also according to manufacturer's instructions. These blots contained polyadenylated RNA obtained from tissues of adult humans who were apparently free of disease. Autoradiography with the labelled blots revealed that VEGF-D mRNA was most abundant in heart, lung and skeletal muscle. VEGF-D mRNA was of intermediate abundance in spleen, ovary, small intestine and colon, and was of low abundance in kidney, pancreas, thymus, prostate and testis. No VEGF-D mRNA was detected in RNA from brain, placenta, liver or peripheral blood leukocytes. In most of the tissues where VEGF-D mRNA was detected the size of the transcript was 2.3 kb. The only exception was skeletal muscle, where two VEGF-D transcripts of 2.3 kb and 2.8 kb were detected. In skeletal muscle the 2.3 kb transcript was more abundant than the 2.8 kb transcript.

Gene expression in mouse embryos

In order to generate an antisense RNA probe for mouse VEGF-D mRNA, the mouse VEGF-D2 cDNA shown in Figure 7 (SEQ ID NO. 7) was inserted into the transcription vector pBluescriptIIKS+ (Stratagene). The resulting plasmid was digested to completion with the restriction endonuclease *Fok*I and then used as template for an *in vitro* transcription reaction with T3 RNA polymerase. This transcription reaction gave rise to an antisense RNA probe for VEGF-D mRNA which was complementary in sequence to the region of the VEGF-D2 cDNA (Figure 7) from the 3'-terminus to the *Fok*I cleavage site closest to the 3'-terminus (nucleotides 1135 to 700). This antisense RNA probe was hybridized at high stringency with paraffin-embedded tissue sections generated from mouse embryos at post-coital day 15.5. Hybridization and washing were essentially as described previously (Achen *et al.*, 1995).

After washing and drying, slides were exposed to autoradiography film for six days.

Development of the autoradiography film revealed that VEGF-D mRNA is localised in the developing lung of post-coital day 15.5 embryos. The signal for VEGF-D mRNA in the lung was strong and highly specific. Control hybridizations with sense probe gave no detectable background in lung or any other tissue.

Summary

The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. Strongest expression was detected in heart, lung and skeletal muscle. In mouse embryos at post-coital day 15.5, strong and specific expression of the VEGF-D gene was detected in the lung. These data suggest that VEGF-D may play a role in lung development, and that expression of the VEGF-D gene in lung persists in the adult, at least in humans. Expression of the gene in other tissues in the adult human suggests that VEGF-D may fulfill other functions in other adult tissues.

Example 13 VEGF-D is Mitogenic for Endothelial Cells

Some members of the VEGF family of proteins, namely VEGF-A (Leung *et al*, 1989) and VEGF-B (Olofsson *et al*, 1996), are mitogenic for endothelial cells. In order to test the mitogenic capacity of VEGFDΔNΔC for endothelial cells, this protein was expressed and purified by affinity chromatography as described in Example 9. Fraction #3, eluted from the M2 affinity column, which contained VEGFDΔNΔC, was diluted 1 in 10 in cell culture medium containing 5% serum and applied to bovine aortic endothelial cells (BAEs) which had been propagated in medium containing 10% serum. The BAEs had been seeded in 24-well dishes at a density of 10,000 cells per well the day before addition of VEGFDΔNΔC, and 3 days after addition of this polypeptide the cells were dissociated with trypsin and counted. Purified VEGF-A was included in

the experiment as positive control. Results are shown in Figure 16. The addition of fraction #3 to the cell culture medium led to a 2.4-fold increase in the number of BAEs after 3 days of incubation, a result which was comparable to that obtained with VEGF-A. Clearly VEGFDΔNΔC is mitogenic for endothelial cells.

Example 14 Localization of the VEGF-D Gene on Human Chromosomes

In order to generate hybridization probes for localization of the VEGF-D gene on human chromosomes, a human genomic DNA clone for VEGF-D was isolated from a human genomic DNA library (Clontech). The genomic library was screened by hybridization with the human VEGF-D cDNA shown in Figure 4, using standard methods (Sambrook *et al.*, 1989). One of the clones thus isolated was shown to contain part of the VEGF-D gene by hybridization to numerous oligonucleotides which were derived in sequence from the human VEGF-D cDNA. A region of the genomic clone, approximately 13 kb in size, was purified from agarose gel, labelled by nick-translation with biotin-14-dATP and hybridized *in situ* at a final concentration of 20 ng/μl to metaphases from two normal human males. The fluorescence *in situ* hybridization (FISH) method was modified from that previously described (Callen *et al*, 1990) in that chromosomes were stained before analysis with propidium iodide (as counterstain) and DAPI (for chromosome identification). Images of metaphase preparations were captured by a cooled CCD camera, using the CytoVision Ultra image collection and enhancement system (Applied Imaging Int. Ltd.). FISH signals and the DAPI banding pattern were merged for analysis.

Fifteen metaphases from the first normal male were examined for fluorescent signal. Ten of the metaphases showed signal on one chromatid (3 cells) or both chromatids (7 cells) of the X chromosome in band p22.1.

There was a total of 9 non-specific background dots observed in these 15 metaphases. A similar result was obtained from hybridization of the probe to 15 metaphases from the second normal male, where signal was observed at Xp22.1 on one chromatid in 7 cells and on both chromatids in 4 cells. In conclusion, the human VEGF-D gene is located on the X chromosome in band p22.1.

Example 15 Localization of the murine VEGF-D Gene on Mouse Chromosomes

The mouse chromosomal location of the VEGF-D gene was determined by interspecific backcross analysis using progeny generated by mating (C57BL/6J x *Mus spretus*)F1 females and CB7BL/67 males as described previously (Copeland and Jenkins, 1991). This interspecific backcross mapping panel has been typed for over 2400 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a 1.3 kb mouse VEGF-D cDNA probe essentially as described (Jenkins *et al.* 1982). Fragments of 7.1, 6.3, 4.7, 2.5 and 2.2 kb were detected in *Taq*I-digested C57BL/6J DNA and major fragments of 7.1, 3.7, 2.7 and 2.2 kb were detected in *Taq*I-digested *M. spretus* DNA. The presence or absence of the 3.7 and 2.7 *Taq*I *M. spretus*-specific fragments, which cosegregated, was followed in backcross mice. The mapping results indicated that the VEGF-D gene is located in the distal region of the mouse X chromosome linked to *Bik*, *DxPasI* and *Ptmb4*. Although 89 mice were analyzed for every marker, up to 133 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total

number of mice analyzed for each pair of loci and the most likely gene order are: centromere - *Btk* - 14/121 - *DxPasI* - 3/99 - VEGF-D - 5/133 - *Ptmb4*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error], calculated using Map Manager (version 2.6.5), are - *Btk* - 11.6+/—2.9 - *DxPasI* - 3.0+/-1.7 - VEGF-D - 3.8+/-1.7 - *Ptmb4*. A description of the probes and RFLPs for the loci linked to the VEGF-D gene, including *Btk*, *DxPasI* and *Ptmb4*, has been reported previously (Hacfliger *et al.*, 1992; Holloway *et al.*, 1997).

We have compared our interspecific map of the X chromosome with a composite mouse linkage map that reports the map location of many uncloned mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Library, Bar Harbor, ME). The VEGF-D gene mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in the locus for an endothelial cell mitogen. The distal region of the mouse X-chromosome shares a region of homology with the short arm of the human X chromosomes (Mouse Genome Database). The placement of the VEGF-D gene in this interval in mouse suggests that the human homolog will map to Xp22. This is consistent with our FISH analysis which has localized the human gene to Xp22.1.

Numerous disease states are caused by mutations in unknown genes which have been mapped to Xp22.1 and the positions immediately surrounding this region in the human. These disease states include Kallmann syndrome, ocular albinism (Nettleship-Falls type), ocular albinism and sensorineural deafness, Partington syndrome, spondyloepiphyseal dysplasia (late), retinitis pigmentosa 15, gonadal dysgenesis (XY female type), Nance-Horan cataract-dental syndrome, retinoschisis, Charcot-Marie-Tooth disease, F-cell production, hypomagnesemia, keratosis follicularis spinulosa decalvans, Coffin-Lowry

syndrome, corneal dermoids, hypophosphatemia, agammaglobulinemia, Aicardi symdrome, hereditary hypophosphatemia II, mental retardation (non-dysmorphic), Opitz G syndrome, pigment disorder (reticulate), dosage-sensitive sex reversal, adrenal hypoplasia, retinitis pigmentosa-6, deafness 4 (congenital sensorineural) and Wilson-Turner syndrome. The positions of the genes involved in these disease states are documented in the OMIM gene map which is edited by Dr. Victor McKusick and colleagues at Johns Hopkins University (USA).

BIOASSAYS TO DETERMINE THE FUNCTION OF VEGF-D

Other assays are conducted to evaluate whether VEGF-D has similar activities to VEGF in relation to endothelial cell function, angiogenesis and wound healing. Further assays may also be performed, depending on the results of receptor binding distribution studies.

I. Assays of Endothelial Cell Function

a) *Endothelial cell proliferation*

Endothelial cell growth assays are performed by methods well known in the art, *eg.* those of Ferrara & Henzel (1989), Gospodarowicz *et al* (1989), and/or Claffey *et al*, Biochim. Biophys. Acta, 1995 1246 1-9.

b) *Cell adhesion assay*

The effect of VEGF-D on adhesion of polmorphonuclear granulocytes to endothelial cells is tested.

c) *Chemotaxis*

The standard Boyden chamber chemotaxis assay is used to test the effect of VEGF-D on chemotaxis.

d) *Plasminogen activator assay*

Endothelial cells are tested for the effect of VEGF-D on plasminogen activator and plasminogen activator

inhibitor production, using the method of Pepper *et al* (1991).

e) *Endothelial cell Migration assay*

The ability of VEGF-D to stimulate endothelial cells to migrate and form tubes is assayed as described in Montesano *et al* (1986). Alternatively, the three-dimensional collagen gel assay described by Joukov *et al* (1996) or a gelatinized membrane in a modified Boyden chamber (Glaser *et al*, 1980) may be used.

II Angiogenesis Assay

The ability of VEGF-D to induce an angiogenic response in chick chorioallantoic membrane is tested as described in Leung *et al* (1989). Alternatively the rat cornea assay of Rastinejad *et al* (1989) may be used; this is an accepted method for assay of *in vivo* angiogenesis, and the results are readily transferrable to other *in vivo* systems.

III Wound Healing

The ability of VEGF-D to stimulate wound healing is tested in the most clinically relevant model available, as described in Schilling *et al* (1959) and utilised by Hunt *et al* (1967).

IV The Haemopoietic System

A variety of *in vitro* and *in vivo* assays using specific cell populations of the haemopoietic system are known in the art, and are outlined below. In particular a variety of *in vitro* murine stem cell assays using fluorescence-activated cell sorter purified cells are particularly convenient:

a) *Repopulating Stem Cells*

These are cells capable of repopulating the bone marrow of lethally irradiated mice, and have the Lin^-,

Rh^{hl}, Ly-6A/E^+, c-kit^+ phenotype. VEGF-D is tested on these cells either alone, or by co-incubation with other factors, followed by measurement of cellular proliferation by ^3H-thymidine incorporation.

b) *Late Stage Stem Cells*

These are cells that have comparatively little bone marrow repopulating ability, but can generate D13 CFU-S. These cells have the Lin^-, Rh^{hl}, Ly-6A/E^+, c-kit^+ phenotype. VEGF-D is incubated with these cells for a period of time, injected into lethally irradiated recipients, and the number of D13 spleen colonies enumerated.

c) *Progenitor-Enriched Cells*

These are cells that respond *in vitro* to single growth factors and have the Lin^-, Rh^{hl}, Ly-6A/E^+, c-kit^+ phenotype. This assay will show if VEGF-D can act directly on haemopoietic progenitor cells. VEGF-D is incubated with these cells in agar cultures, and the number of colonies present after 7-14 days is counted.

V Atherosclerosis

Smooth muscle cells play a crucial role in the development or initiation of atherosclerosis, requiring a change of their phenotype from a contractile to a synthetic state. Macrophages, endothelial cells, T lymphocytes and platelets all play a role in the development of atherosclerotic plaques by influencing the growth and phenotypic modulations of smooth muscle cell. An *in vitro* assay using a modified Rose chamber in which different cell types are seeded on to opposite coverslips measures the proliferative rate and phenotypic modulations of smooth muscle cells in a multicellular environment, and is used to assess the effect of VEGF-D on smooth muscle cells.

VI M tastasis

The ability of VEGF-D to inhibit metastasis is assayed using the Lewis lung carcinoma model, for example using the method of Cao *et al* (1995).

VII VEGF-D in Other Cell Types

The effects of VEGF-D on proliferation, differentiation and function of other cell types, such as liver cells, cardiac muscle and other cells, endocrine cells and osteoblasts can readily be assayed by methods known in the art, such as ^{3}H-thymidine uptake by *in vitro* cultures. Expression of VEGF-D in these and other tissues can be measured by techniques such as Northern blotting and hybridization or by *in situ* hybridization.

VIII Construction of VEGF-D Variants and Analogues

VEGF-D is a member of the PDGF family of growth factors which exhibits a high degree of homology to the other members of the PDGF family. VEGF-D contains eight conserved cysteine residues which are characteristic of this family of growth factors. These conserved cysteine residues form intra-chain disulfide bonds which produce the cysteine knot structure, and inter-chain disulfide bonds that form the protein dimers which are characteristic of members of the PDGF family of growth factors. VEGF-D will interact with protein tyrosine kinase growth factor receptors.

In contrast to proteins where little or nothing is known about the protein structure and active sites needed for receptor binding and consequent activity, the design of active mutants of VEGF-D is greatly facilitated by the fact that a great deal is known about the active sites and important amino acids of the members of the PDGF family of growth factors.

Published articles elucidating the structure/activity relationships of members of the PDGF family of growth factors include for PDGF: Oestman *et al*,

J. Biol. Chem., 1991 266 10073-10077; Andersson *et al*, J. Biol. Chem., 1992 267 11260-1266; Oefner *et al*, EMBO J., 1992 11 3921-3926; Flemming *et al*, Molecular and Cell Biol., 1993 13 4066-4076 and Andersson *et al*, Growth Factors, 1995 12 159-164; and for VEGF: Kim *et al*, Growth Factors, 1992 7 53-64; Pötgens *et al*, J. Biol. Chem., 1994 269 32879-32885 and Claffey *et al*, Biochem. Biophys. Acta, 1995 1246 1-9. From these publications it is apparent that because of the eight conserved cysteine residues, the members of the PDGF family of growth factors exhibit a characteristic knotted folding structure and dimerization, which result in formation of three exposed loop regions at each end of the dimerized molecule, at which the active receptor binding sites can be expected to be located.

Based on this information, a person skilled in the biotechnology arts can design VEGF-D mutants with a very high probability of retaining VEGF-D activity by conserving the eight cysteine residues responsible for the knotted folding arrangement and for dimerization, and also by conserving, or making only conservative amino acid substitutions in the likely receptor sequences in the loop 1, loop 2 and loop 3 region of the protein structure.

The formation of desired mutations at specifically targeted sites in a protein structure is considered to be a standard technique in the arsenal of the protein chemist (Kunkel *et al*, Methods in Enzymol., 1987 154 367-382). Examples of such site-directed mutagenesis with VEGF can be found in Pötgens *et al*, J. Biol. Chem., 1994 269 32879-32885 and Claffey *et al*, Biochim. Biophys. Acta, 1995 1246 1-9. Indeed, site-directed mutagenesis is so common that kits are commercially available to facilitate such procedures (*eg*. Promega 1994-1995 Catalog., Pages 142-145).

The endothelial cell proliferating activity of VEGF-D mutants can be readily confirmed by well established screening procedures. For example, a

procedure analogous to the endothelial cell mitotic assay described by Claffey *et al*, (Biochim. Biophys. Acta., 1995 1246 1-9) can be used. Similarly the effects of VEGF-D on proliferation of other cell types, on cellular differentiation and on human metastasis can be tested using methods which are well known in the art.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LUDWIG INSTITUTE FOR CANCER RESEARCH

(ii) TITLE OF INVENTION: GROWTH FACTOR

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 1200 G Street, NW, Suite 700
(C) CITY: Washington
(D) STATE: DC
(E) COUNTRY: United States of America
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: EVANS, Joseph D.
(C) REFERENCE/DOCKET NUMBER: 1064/42983PCT

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (202) 628-8844
(C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2846 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Human Breast

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGAATTCAGT GAAGTAAGAA AGACAAAGTG TTCATTGGAG ATTTTTAGTA AGGGGCCAAC 60

AGAGCTGCTA AAGTCATGCT TCACTTAACG ATGGGGATAT GTTCGGAGAA ATGCATTGTT 120

AGGTGATTTT GTCGTTGTGC AAGCATCTTA GAGTACACTT AGACAAACCT AGCTGGTATA 180

ACCTAGGTGT GTAGTAGGAT ATATGGTATA GCCTATTGTT CCTAGGCTAC AAACCCATAC 240

AGCATGTTCC TGTACTGAAT ACTGAGGCAA CTGCAACACC GTGGTGAGTA TTTGTGTATC 300

TAAACATACC TAAACATAGA AAAGATACAG TAAAAATATG GCATTATAGT CTTATGGGAC 360

TACTGTCATA CATACAGTCC ATATATTGTT GACTGTGTAA TGTTGACCTG AATGTCATTA 420

TGTGGCAGGC ACATGACTGT GTCGCTAACC TTTGCACAAG ATTACTGTAG GATTACATGA 480

GATAGTTGTA AATAATTGGT GGGGTACTGG GCACCTAGTA GGTATGCATA CATGTTCACC 540

ATCATTATGG TTGTTTTAAA TCACCTAACC CAGGCCCTGC ACATAGTAAG ACATCAACAA 600

ATTGTAGCTG CTACTATTTT GCGCATCTAA TCTTAATATC ATTTATTTTG TAGTCCTTGG 660

ATGTTCCCTC CTTTATGACT TCTTTTTTTT TTGTTGTCCT TCCTTTAGCC CTCCATCCTC 720

TACAGCTCAG CATCAGAACA CTCTCTTTTT AGACTCCGAT ATGGGGTCCT CCAAGAAAGT 780

TACTCTCTCA GTGCTCAGCC GGGAGCAGTC GGAAGGGGTT GGAGCGAGGG TCCGGAGAAG 840

CATTGGCAGA CCCGAGTTAA AAAATCTGGA TCCGTTTTTA CTGTTTGATG AATTTAAAGG 900

AGGTAGACCA GGAGGATTTC CTGATCATCC ACATCGAGGT TTTGAAACAG TATCCTACCT 960

CCTGGAAGGG GGCAGCATGG CCCATGAAGA CTTCTGTGGA CACACTGGTA AAATGAACCC 1020

AGGAGATTTG CAGTGGATGA CTGCGGGCCG GGGCATTCTG CACGCTGAGA TGCCTTGCTC 1080

AGAGGAGCCA GCCCATGGCC TACAACTGTG GGTTAATTTG AGGAGCTCAG AGAAGATGGT 1140

GGAGCCTCAG TACCAGGAAC TGAAAAGTGA AGAAATCCCT AAACCCAGTA AGGATGGTGT 1200

GACAGTTGCT GTCATTTCTG GAGAAGCCCT GGGAATAAAG TCCAAGGTTT ACACTCGCAC 1260

ACCAACCTTA TATTTGGACT TCAAATTGGA CCCAGGAGCC AAACATTCCC AACCTATCCC 1320

TAAAGGGTGG ACAAGCTTCA TTTACACGAT ATCTGGAGAT GTGTATATTG CCCTCTCTAT 1380

ATCCCAGCAC AGGTATGCCC AGGGCAGGGT GCCTTTCAGC TTACAGAACA TTCAGTGAGG 1440

GAAGAGAATA TGAACACCAG TCATGACACA TCCTGTGCAC AGATGAAAGT CCAGGCACCA 1500

TTATGTGTTT TGATACCTCG CTAAGACGTT GGCAACCTCC ATACTGATAA AGGGATGGAG 1560

CTACAGTGGA CTCCAAGGGG AGCAGGAATC TGCCTATCTC CTGGGAGAAG GAAATGGAAG 1620

GAGGGCCCGA TGATGCACAA CAAAAAATAG AACCTCATCA CACAGCAGTG CTTGGAGAAG 1680

GTGACAGTGT CCAAGTGGAG AACAAGGATC CCAAGAGAAG CCACTTTGTC TTAATTGCTG 1740

GGGAGCCATT AAGAGAACCA GTTATCCAAC ATGCGATCAT CTCAGTCCAC ATTGGAACGA 1800

TCTGAACAGC AGATCAGGGC TGCTTCTAGT TTGGAGGAAC TACTTCGAAT TACTCACTCT 1860

GAGGACTGGA AGCTGTGGAG ATGCAGGCTG AGGCTCAAAA GTTTTACCAG TATGGACTCT 1920

CGCTCAGCAT CCCATCGGTC CACTAGGTTT GCGGCAACTT TCTATGACAT TGAAACACTA 1980

AAAGTTATAG ATGAAGAATG GCAAAGAACT CAGTGCAGCC CTAGAGAAAC GTGCGTGGAG 2040

GTGGCCAGTG AGCTGGGGAA GAGTACCAAC ACATTCTTCA AGCCCCCTTG TGTGAACGTG 2100

TTCCGATGTG GTGGCTGTTG CAATGAAGAG AGCCTTATCT GTATGAACAC CAGCACCTCG 2160

TACATTTCCA AACAGCTCTT TGAGATATCA GTGCCTTTGA CATCAGTACC TGAATTAGTG 2220

CCTGTTAAAG TTGCCAATCA TACAGGTTGT AAGTGCTTGC CAACAGCCCC CCGCCATCCA 2280

TACTCAATTA TCAGAAGATC CATCCAGATC CCTGAAGAAG ATCGCTGTTC CCATTCCAAG 2340

AAACTCTGTC CTATTGACAT GCTATGGGAT AGCAACAAAT GTAAATGTGT TTTGCAGGAG 2400

GAAAATCCAC TCGCTGGAAC AGAAGACCAC TCTCATCTCC AGGAACCAGC TCTCTGTGGG 2460

CCACACATGA TGTTTGACGA AGATCGTTGC GAGTGTGTCT GTAAAACACC ATGTCCCAAA 2520

GATCTAATCC AGCACCCCAA AAACTGCAGT TGCTTTGAGT GCAAAGAAAG TCTGGAGACC 2580

TGCTGCCAGA AGCACAAGCT ATTTCACCCA GACACCTGCA GCTGTGAGGA CAGATGCCCC 2640

TTTCATACCA GACCATGTGC AAGTGGCAAA ACAGCATGTG CAAAGCATTG CCGCTTTCCA 2700

AAGGAGAAAA GGGCTGCCCA GGGGCCCCAC AGCCGAAAGA ATCCTTGATT CAGCGTTCCA 2760

AGTTCCCCAT CCCTGTCATT TTTAACAGCA TGCTGCTTTG CCAAGTTGCT GTCACTGTTT 2820

TTTTCCCAGG TGTTAAAAAA AAAAAA 2846

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Xaa Cys Val Xaa Xaa Xaa Arg Cys Xaa Gly Cys Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 325 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Human Breast

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Arg Ser Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg
1 5 10 15

Ala Ala Ser Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp
20 25 30

Trp Lys Leu Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met
35 40 45

Asp Ser Arg Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe
50 55 60

Tyr Asp Ile Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr
65 70 75 80

```
Gln Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly
                85                  90                  95

Lys Ser Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg
            100                 105                 110

Cys Gly Gly Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser
        115                 120                 125

Thr Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr
    130                 135                 140

Ser Val Pro Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys
145                 150                 155                 160

Lys Cys Leu Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg
                165                 170                 175

Ser Ile Gln Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu
            180                 185                 190

Cys Pro Ile Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu
        195                 200                 205

Gln Glu Glu Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln
    210                 215                 220

Glu Pro Ala Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys
225                 230                 235                 240

Glu Cys Val Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro
                245                 250                 255

Lys Asn Cys Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys
            260                 265                 270

Gln Lys His Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg
        275                 280                 285

Cys Pro Phe His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala
    290                 295                 300

Lys His Cys Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His
305                 310                 315                 320

Ser Arg Lys Asn Pro
                325
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2029 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Human Lung

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GTTGGGTTCC AGCTTTCTGT AGCTGTAAGC ATTGGTGGCC ACACCACCTC CTTACAAAGC      60
```

AACTAGAACC TGCGGCATAC ATTGGAGAGA TTTTTTTAAT TTTCTGGACA TGAAGTAAAT 120

TTAGAGTGCT TTCTAATTTC AGGTAGAAGA CATGTCCACC TTCTGATTAT TTTTGGAGAA 180

CATTTTGATT TTTTTCATCT CTCTCTCCCC ACCCCTAAGA TTGTGCAAAA AAAGCGTACC 240

TTGCCTAATT GAAATAATTT CATTGGATTT TGATCAGAAC TGATTATTTG GTTTTCTGTG 300

TGAAGTTTTG AGGTTTCAAA CTTTCCTTCT GGAGAATGCC TTTTGAAACA ATTTTCTCTA 360

GCTGCCTGAT GTCAACTGCT TAGTAATCAG TGGATATTGA AATATTCAAA ATGTACAGAG 420

AGTGGGTAGT GGTGAATGTT TTCATGATGT TGTACGTCCA GCTGGTGCAG GGCTCCAGTA 480

ATGAACATGG ACCAGTGAAG CGATCATCTC AGTCCACATT GGAACGATCT GAACAGCAGA 540

TCAGGGCTGC TTCTAGTTTG GAGGAACTAC TTCGAATTAC TCACTCTGAG GACTGGAAGC 600

TGTGGAGATG CAGGCTGAGG CTCAAAAGTT TTACCAGTAT GGACTCTCGC TCAGCATCCC 660

ATCGGTCCAC TAGGTTTGCG GCAACTTTCT ATGACATTGA AACACTAAAA GTTATAGATG 720

AAGAATGGCA AAGAACTCAG TGCAGCCCTA GAGAAACGTG CGTGGAGGTG GCCAGTGAGC 780

TGGGGAAGAG TACCAACACA TTCTTCAAGC CCCCTTGTGT GAACGTGTTC CGATGTGGTG 840

GCTGTTGCAA TGAAGAGAGC CTTATCTGTA TGAACACCAG CACCTCGTAC ATTTCCAAAC 900

AGCTCTTTGA GATATCAGTG CCTTTGACAT CAGTACCTGA ATTAGTGCCT GTTAAAGTTG 960

CCAATCATAC AGGTTGTAAG TGCTTGCCAA CAGCCCCCCG CCATCCATAC TCAATTATCA 1020

GAAGATCCAT CCAGATCCCT GAAGAAGATC GCTGTTCCCA TTCCAAGAAA CTCTGTCCTA 1080

TTGACATGCT ATGGGATAGC AACAAATGTA AATGTGTTTT GCAGGAGGAA AATCCACTTG 1140

CTGGAACAGA AGACCACTCT CATCTCCAGG AACCAGCTCT CTGTGGGCCA CACATGATGT 1200

TTGACGAAGA TCGTTGCGAG TGTGTCTGTA AAACACCATG TCCCAAAGAT CTAATCCAGC 1260

ACCCCAAAAA CTGCAGTTGC TTTGAGTGCA AAGAAAGTCT GGAGACCTGC TGCCAGAAGC 1320

ACAAGCTATT TCACCCAGAC ACCTGCAGCT GTGAGGACAG ATGCCCCTTT CATACCAGAC 1380

CATGTGCAAG TGGCAAAACA GCATGTGCAA AGCATTGCCG CTTTCCAAAG GAGAAAAGGG 1440

CTGCCCAGGG GCCCCACAGC CGAAAGAATC CTTGATTCAG CGTTCCAAGT TCCCCATCCC 1500

TGTCATTTTT AACAGCATGC TGCTTTGCCA AGTTGCTGTC ACTGTTTTTT TCCCAGGTGT 1560

TAAAAAAAAA ATCCATTTTA CACAGCACCA CAGTGAATCC AGACCAACCT TCCATTCACA 1620

CCAGCTAAGG AGTCCCTGGT TCATTGATGG ATGTCTTCTA GCTGCAGATG CCTCTGCGCA 1680

CCAAGGAATG GAGAGGAGGG GACCCATGTA ATCCTTTTGT TTAGTTTTGT TTTTGTTTTT 1740

TGGTGAATGA GAAAGGTGTG CTGGTCATGG AATGGCAGGT GTCATATGAC TGATTACTCA 1800

GAGCAGATGA GGAAAACTGT AGTCTCTGAG TCCTTTGCTA ATCGCAACTC TTGTGAATTA 1860

TTCTGATTCT TTTTTATGCA GAATTTGATT CGTATGATCA GTACTGACTT TCTGATTACT 1920

GTCCAGCTTA TAGTCTTCCA GTTTAATGAA CTACCATCTG ATGTTTCATA TTTAAGTGTA 1980

TTTAAAGAAA ATAAACACCA TTATTCAAGC CAAAAAAAAA AAAAAAAAA 2029

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 354 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Human Lung

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Tyr Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val
1               5                  10                  15

Gln Leu Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser
            20                  25                  30

Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
        35                  40                  45

Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu
    50                  55                  60

Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg
65                  70                  75                  80

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile
                85                  90                  95

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
            100                 105                 110

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr
        115                 120                 125

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
    130                 135                 140

Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr
145                 150                 155                 160

Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
                165                 170                 175

Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu
            180                 185                 190

Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
        195                 200                 205

Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile
    210                 215                 220

Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu
225                 230                 235                 240

Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala
                245                 250                 255

Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val
            260                 265                 270
```

```
Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys
        275                 280                 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His
    290                 295                 300

Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe
305                 310                 315                 320

His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys
                325                 330                 335

Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys
            340                 345                 350

Asn Pro
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1325 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Mouse Lung

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
GGAGAATGCC TTTTGCAACA CTTTTCAGTA GCTGCCTGGA AACAACTGCT TAGTCATCGG      60

TAGACATTTA AAATATTCAA AATGTATGGA GAATGGGGAA TGGGGAATAT CCTCATGATG     120

TTCCATGTGT ACTTGGTGCA GGGCTTCAGG AGCGAACATG GACCAGTGAA GGATTTTTCT     180

TTTGAGCGAT CATCCCGGTC CATGTTGGAA CGATCTGAAC AACAGATCCG AGCAGCTTCT     240

AGTTTGGAGG AGTTGCTGCA AATCGCGCAC TCTGAGGACT GGAAGCTGTG GCGATGCCGG     300

TTGAAGCTCA AAAGTCTTGC CAGTATGGAC TCACGCTCAG CATCCCATCG CTCCACCAGA     360

TTTGCGGCAA CTTTCTATGA CACTGAAACA CTAAAAGTTA TAGATGAAGA ATGGCAGAGG     420

ACCCAATGCA GCCCTAGAGA GACATGCGTA GAAGTCGCCA GTGAGCTGGG GAAGACAACC     480

AACACATTCT TCAAGCCCCC CTGTGTAAAT GTCTTCCGGT GTGGAGGCTG CTGCAACGAA     540

GAGGGTGTGA TGTGTATGAA CACAAGCACC TCCTACATCT CCAAACAGCT CTTTGAGATA     600

TCAGTGCCTC TGACATCAGT GCCCGAGTTA GTGCCTGTTA AAATTGCCAA CCATACGGGT     660

TGTAAGTGCT TGCCCACGGG CCCCCGCCAT CCTTACTCAA TTATCAGAAG ATCCATTCAG     720

ACCCCAGAAG AAGATGAATG TCCTCATTCC AAGAAACTCT GTCCTATTGA CATGCTGTGG     780

GATAACACCA AATGTAAATG TGTTTTGCAA GACGAGACTC CACTGCCTGG GACAGAAGAC     840

CACTCTTACC TCCAGGAACC CACTCTCTGT GGACCGCACA TGACGTTTGA TGAAGATCGC     900

TGTGAGTGCG TCTGTAAAGC ACCATGTCCG GGAGATCTCA TTCAGCACCC GGAAAACTGC     960
```

AGTTGCTTTG AGTGCAAAGA AAGTCTGGAG AGCTGCTGCC AAAAGCACAA GATTTTTCAC 1020

CCAGACACCT GCAGCTGTGA GGACAGATGT CCTTTTCACA CCAGAACATG TGCAAGTAGA 1080

AAGCCAGCCT GTGGAAAGCA CTGGCGCTTT CCAAAGGAGA CAAGGGCCCA GGGACTCTAC 1140

AGCCAGGAGA ACCCTTGATT CAACTTCCTT TCAAGTCCCC CCATCTCTGT CATTTTAAAC 1200

AGCTCACTGC TTTGTCAAGT TGCTGTCACT GTTGCCCACT ACCCCTTGAA CATGTGCAAA 1260

CACAGACACA CACACACACA CACACACAGA GCAACTAGAA TTATGTTTTC TAGGTGCTGC 1320

CTAAG 1325

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1135 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Mouse Lung

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAACTTTGCT TCTGGAGAAT GCCTTTTGCA ACACTTTTCA GTAGCTGCCT GGAAACAACT 60

GCTTAGTCAT CGGTAGACAT TTAAAATATT CAAAATGTAT GGAGAATGGG GAATGGGGAA 120

TATCCTCATG ATGTTCCATG TGTACTTGGT GCAGGGCTTC AGGAGCGAAC ATGGACCAGT 180

GAAGCGATCA TCCCGGTCCA TGTTGGAACG ATCTGAACAA CAGATCCGAG CAGCTTCTAG 240

TTTGGAGGAG TTGCTGCAAA TCGCGCACTC TGAGGACTGG AAGCTGTGGC GATGCCGGTT 300

GAAGCTCAAA AGTCTTGCCA GTATGGACTC ACGCTCAGCA TCCCATCGCT CCACCAGATT 360

TGCGGCAACT TTCTATGACA CTGAAACACT AAAAGTTATA GATGAAGAAT GGCAGAGGAC 420

CCAATGCAGC CCTAGAGAGA CATGCGTAGA AGTCGCCAGT GAGCTGGGGA AGACAACCAA 480

CACATTCTTC AAGCCCCCCT GTGTAAATGT CTTCCGGTGT GGAGGCTGCT GCAACGAAGA 540

GGGTGTGATG TGTATGAACA CAAGCACCTC CTACATCTCC AAACAGCTCT TTGAGATATC 600

AGTGCCTCTG ACATCAGTGC CCGAGTTAGT GCCTGTTAAA ATTGCCAACC ATACGGGTTG 660

TAAGTGCTTG CCCACGGGCC CCCGCCATCC TTACTCAATT ATCAGAAGAT CCATTCAGAC 720

CCCAGAAGAA GATGAATGTC CTCATTCCAA GAAACTCTGT CCTATTGACA TGCTGTGGGA 780

TAACACCAAA TGTAAATGTG TTTTGCAAGA CGAGACTCCA CTGCCTGGGA CAGAAGACCA 840

CTCTTACCTC CAGGAACCCA CTCTCTGTGG ACCGCACATG ACGTTTGATG AAGATCGCTG 900

TGAGTGCGTC TGTAAAGCAC CATGTCCGGG AGATCTCATT CAGCACCCGG AAAACTGCAG 960

TTGCTTTGAG TGCAAAGAAA GTCTGGAGAG CTGCTGCCAA AAGCACAAGA TTTTTCACCC 1020

AGACACCTGC AGGTCAATGG TCTTTTCGCT TTCCCCTTAA CTTGGTTTAC TGATGACATT 1080

TAAAGGACAT ACTAATCTGA TCTGTTCAGG CTCTTTTCTC TCAGAGTCCA AGCAC 1135

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 358 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Mouse Lung

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Tyr Gly Glu Trp Gly Met Gly Asn Ile Leu Met Met Phe His Val
1 5 10 15

Tyr Leu Val Gln Gly Phe Arg Ser Glu His Gly Pro Val Lys Asp Phe
20 25 30

Ser Phe Glu Arg Ser Ser Arg Ser Met Leu Glu Arg Ser Glu Gln Gln
35 40 45

Ile Arg Ala Ala Ser Ser Leu Glu Glu Leu Leu Gln Ile Ala His Ser
50 55 60

Glu Asp Trp Lys Leu Trp Arg Cys Arg Leu Lys Leu Lys Ser Leu Ala
65 70 75 80

Ser Met Asp Ser Arg Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala
85 90 95

Thr Phe Tyr Asp Thr Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln
100 105 110

Arg Thr Gln Cys Ser Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu
115 120 125

Leu Gly Lys Thr Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val
130 135 140

Phe Arg Cys Gly Gly Cys Cys Asn Glu Glu Gly Val Met Cys Met Asn
145 150 155 160

Thr Ser Thr Ser Tyr Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro
165 170 175

Leu Thr Ser Val Pro Glu Leu Val Pro Val Lys Ile Ala Asn His Thr
180 185 190

Gly Cys Lys Cys Leu Pro Thr Gly Pro Arg His Pro Tyr Ser Ile Ile
195 200 205

Arg Arg Ser Ile Gln Thr Pro Glu Glu Asp Glu Cys Pro His Ser Lys
210 215 220

Lys Leu Cys Pro Ile Asp Met Leu Trp Asp Asn Thr Lys Cys Lys Cys
225 230 235 240

Val Leu Gln Asp Glu Thr Pro Leu Pro Gly Thr Glu Asp His Ser Tyr
245 250 255

Leu Gln Glu Pro Thr Leu Cys Gly Pro His Met Thr Phe Asp Glu Asp
260 265 270

Arg Cys Glu Cys Val Cys Lys Ala Pro Cys Pro Gly Asp Leu Ile Gln
275 280 285

His Pro Glu Asn Cys Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Ser
290 295 300

Cys Cys Gln Lys His Lys Ile Phe His Pro Asp Thr Cys Ser Cys Glu
305 310 315 320

Asp Arg Cys Pro Phe His Thr Arg Thr Cys Ala Ser Arg Lys Pro Ala
325 330 335

Cys Gly Lys His Trp Arg Phe Pro Lys Glu Thr Arg Ala Gln Gly Leu
340 345 350

Tyr Ser Gln Glu Asn Pro
355

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 321 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE: Mouse Lung

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Tyr Gly Glu Trp Gly Met Gly Asn Ile Leu Met Met Phe His Val
1 5 10 15

Tyr Leu Val Gln Gly Phe Arg Ser Glu His Gly Pro Val Lys Arg Ser
20 25 30

Ser Arg Ser Met Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
35 40 45

Ser Leu Glu Glu Leu Leu Gln Ile Ala His Ser Glu Asp Trp Lys Leu
50 55 60

Trp Arg Cys Arg Leu Lys Leu Lys Ser Leu Ala Ser Met Asp Ser Arg
65 70 75 80

Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Thr
85 90 95

Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
100 105 110

Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Thr Thr
115 120 125

Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
130 135 140

Cys Cys Asn Glu Glu Gly Val Met Cys Met Asn Thr Ser Thr Ser Tyr
145 150 155 160

Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
165 170 175

Glu Leu Val Pro Val Lys Ile Ala Asn His Thr Gly Cys Lys Cys Leu
180 185 190

Pro Thr Gly Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
195 200 205

Thr Pro Glu Glu Asp Glu Cys Pro His Ser Lys Lys Leu Cys Pro Ile
210 215 220

Asp Met Leu Trp Asp Asn Thr Lys Cys Lys Cys Val Leu Gln Asp Glu
225 230 235 240

Thr Pro Leu Pro Gly Thr Glu Asp His Ser Tyr Leu Gln Glu Pro Thr
245 250 255

Leu Cys Gly Pro His Met Thr Phe Asp Glu Asp Arg Cys Glu Cys Val
260 265 270

Cys Lys Ala Pro Cys Pro Gly Asp Leu Ile Gln His Pro Glu Asn Cys
275 280 285

Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Ser Cys Cys Gln Lys His
290 295 300

Lys Ile Phe His Pro Asp Thr Cys Arg Ser Met Val Phe Ser Leu Ser
305 310 315 320

Pro

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nuleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGCTGCTTC TAGTTTGGAG 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nuleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(vi) ORIGINAL SOURCE:
(F) TISSUE TYPE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTCGCAAC GATCTTCGTC 20

Patent Claims

1. An isolated nucleic acid molecule comprising a nucleic acid sequence which encodes a polypeptide comprising a sequence of amino acids substantially corresponding to the amino acid sequence set out in SEQ ID NO.3, SEQ ID NO.5, SEQ ID NO.8 or SEQ ID NO. 9, said polypeptide having the ability to stimulate vascular permeability or proliferation of endothelial cells, or a fragment or analogue thereof which has the ability to stimulate at least one biological activity selected from the group consisting of angiogenesis, vascular permeability, endothelial cell proliferation, differentiation, migration or survival, or which has the ability to bind to endothelial cells, but is unable to stimulate at least one of said biological activities.

2. A nucleic acid molecule according to claim 1, wherein said nucleic acid molecule comprises a nucleic acid sequence which encodes the amino acid sequence

Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys

(SEQ ID NO.2).

3. A nucleic acid molecule according to claim 1, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

4. A nucleic acid molecule according to claim 1, which is a genomic DNA.

5. A nucleic acid molecule according to claim 1, which is a cDNA.

6. A nucleic acid molecule according to claim 5, which comprises the nucleic acid sequence of SEQ ID NO.1, SEQ ID NO.4, SEQ ID NO.6, SEQ ID NO.7, or a DNA sequence

which hybridizes to one of the foregoing sequences under stringent conditions.

7. A nucleic acid molecule according to claim 6, which comprises the nucleic acid sequence of SEQ ID NO.4.

8. A nucleic acid molecule according to any one of claims 1 to 7, which encodes a polypeptide which has the ability to stimulate vascular permeability or proliferation of endothelial cells.

9. A nucleic acid molecule according to claim 1, which encodes a polypeptide comprising amino acid residues 64 through 172 of SEQ ID NO:3 or amino acid residues 93 through 201 of SEQ ID NO:5.

10. A nucleic acid molecule according to claim 9, wherein said polypeptide further comprises an affinity tag peptide sequence.

11. A nucleic acid molecule according to any one of Claims 1 to 7, which encodes a polypeptide which has the ability to bind to endothelial cells but is unable to stimulate endothelial cell proliferation.

12. A nucleic acid molecule according to claim 11, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

13. A nucleic acid molecule according to claim 1, wherein said nucleic acid molecule is a human DNA molecule.

14. A vector comprising a nucleic acid according to any one of Claims 1 to 13.

15. A host cell transformed or transformed with a vector according to claim 14.

16. An isolated polypeptide which comprises a sequence of amino acids substantially corresponding to the amino acid sequence set out in SEQ ID NO.3, SEQ ID NO.5, SEQ ID NO.8 or SEQ ID NO. 9, said polypeptide having the ability to stimulate vascular permeability or proliferation of endothelial cells, or a fragment or analogue thereof which has the ability to stimulate at least one endothelial cell biological activity selected from the group consisting of cell proliferation, cell differentiation, cell migration, cell survival and vascular permeability, or which has the ability to bind to endothelial cells but is unable to stimulate at least one of said biological activities.

17. A polypeptide according to claim 16, wherein said polypeptide comprises the amino acid sequence
Pro-Xaa-Cys-Val-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys (SEQ ID NO.2).

18. A polypeptide according to claim 16, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

19. A polypeptide according to Claim 16, which comprises a sequence of amino acids substantially corresponding to SEQ ID NO:3 or SEQ ID NO:5.

20. A polypeptide according to Claim 19, which comprises a sequence of amino acids substantially corresponding to SEQ ID NO 5.

21. A polypeptide according to any one of Claims 16 to 20, which has the ability to stimulate proliferation of endothelial cells.

22. A polypeptide according to any one of claims 16 to 20, which has the ability to induce endothelial cell differentiation.

23. A polypeptide according to any one of claims 16 to 20, which has the ability to induce vascular permeability.

24. A polypeptide according to Claim 16, comprising amino acid residues 64 through 172 of SEQ ID NO:3 or 93 through 201 of SEQ ID NO:5.

25. A polypeptide according to claim 24, further comprising an affinity tag peptide sequence.

26. A polypeptide according to claim 16 or 17, which has the ability to bind to endothelial cells but is unable to stimulate proliferation of endothelial cells.

27. A polypeptide according to claim 26, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

28. A polypeptide according to any one of claims 16 to 20, wherein said polypeptide is a human protein.

29. An antibody specifically reactive with a polypeptide according to any one of Claims 16 to 28.

30. An antibody according to claim 29, wherein said antibody is a polyclonal antibody.

31. An antibody according to claim 29, wherein said antibody is a monoclonal antibody.

32. An antibody according to claim 29, wherein said antibody is labelled with a detectable label.

33. A method of making a polypeptide according to claim 16, said method comprising the steps of:

culturing a host cell transformed or transfected with a vector comprising a nucleic acid sequence encoding said polypeptide operably associated with a promoter sequence such that the nucleic acid sequence encoding said polypeptide is expressed; and

isolating said polypeptide from said host cell or from a growth medium in which said host cell is cultured.

34. A method of isolation of VEGF-D comprising the step of exposing a cell which expresses VEGF-D to heparin to facilitate release of VEGF-D from the cell, and purifying the thus-released VEGF-D.

35. A method of making a vector capable of expressing a polypeptide encoded by a nucleic acid molecule according to any one of Claims 1 to 9, said method comprising inserting said nucleic acid molecule into a vector in a position in which said nucleic acid molecule is operatively connected with at least one promoter.

36. A vector comprising an anti-sense nucleotide sequence, said anti-sense nucleotide sequence being complementary to at least a part of a VEGF-D genomic DNA sequence or a VEGF-D RNA sequence or a cDNA sequence which encodes VEGF-D or a fragment or analogue thereof which promotes at least one bioactivity selected from vascular permeability, proliferation of endothelial cells and endothelial cell differentiation, whereby said vector can be used to inhibit said at least one bioactivity.

37. A method of stimulating endothelial cell proliferation comprising contacting endothelial cells with an effective endothelial cell proliferation stimulating amount of a polypeptide according to claim 16.

38. A method according to claim 37, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

39. A method of stimulating at least one bioactivity selected from endothelial cell proliferation, endothelial cell differentiation and vascular permeability, *in vivo* in a mammal, said method comprising administering to said mammal an effective bioactivity stimulating amount of a polypeptide according to claim 16, which has the ability to stimulate said at least one bioactivity.

40. A method according to Claim 39, wherein said polypeptide comprises amino acid residues 64 through 172 of SEQ ID NO:3 or amino acid residues 93 through 201 of SEQ ID NO:5.

41. A method according to Claim 39, wherein lymphatic vessel endothelial cell proliferation is stimulated.

42. A method of stimulating at least one bioactivity selected from angiogenesis and neovascularization in a mammal, said method comprising the step of administering to said mammal an effective angiogenesis or neovascularization stimulating amount of a polypeptide according to claim 16, said polypeptide having the ability to stimulate endothelial cell proliferation.

43. A method according to Claim 42, wherein said polypeptide comprises amino acid residues 64 through 172 of SEQ ID NO:3 or amino acid residues 93 through 201 of SEQ ID NO:5.

44. A method according to claim 43, wherein said polypeptide further comprises an affinity tag peptide sequence.

45. A method according claim 32, further comprising co-administering at least one substance selected from the group consisting of VEGF, VEGF-B, VEGF-C, PlGF, PDGF, FGF and heparin.

46. A method of inhibiting a bioactivity selected from angiogenesis and neovascularization in a mammal, said method comprising the step of administering to said mammal an effective angiogenesis or neovascularization inhibiting amount of a VEGF-D antagonist.

47. A method according to claim 46, wherein said VEGF-D antagonist comprises an antibody specific to VEGF—D.

48. A method according to claim 46, wherein said VEGF-D antagonist comprises a polypeptide which binds to endothelial cells but which is unable to stimulate at least one biological activity selected from proliferation of endothelial cells, endothelial cell differentiation and vascular permeability.

49. A method according to claim 48, wherein said endothelial cells are selected from the group consisting of vascular endothelial cells and lymphatic endothelial cells.

50. A method of inhibiting VEGF-D expression in a mammal comprising the step of transforming target cells expressing VEGF-D with a vector according to Claim 36.

51. A pharmaceutical composition comprising a polypeptide according to any one of claims 16 to 24, and a pharmaceutically acceptable carrier or adjuvant.

52. A pharmaceutical composition according to claim 51, further comprising at least one substance selected from the group consisting of VEGF, VEGF-B, VEGF-C, PlGF, PDGF and heparin.

53. A pharmaceutical composition comprising an antibody according to any one of claims 29 through 32, and a pharmaceutically acceptable carrier or adjuvant.

54. A pharmaceutical composition according to Claim 53, wherein said antibody is a monoclonal antibody.

55. A protein dimer comprising a first polypeptide according to any one of claims 16 to 24, and a second polypeptide.

56. A protein dimer according to Claim 55, wherein said protein dimer is a homodimer in which the second polypeptide is identical to the first polypeptide.

57. A protein dimer according to claim 55, wherein said protein dimer is a heterodimer in which the second polypeptide is selected from VEGF, VEGF-B, VEGF-C, PlGF and PDGF.

58. A method of detecting VEGF-D in a biological sample, comprising the step of contacting the sample with a reagent capable of binding VEGF-D, and detecting the occurrence of binding of said reagent.

59. A method according to claim 58, wherein said reagent comprises an antibody according to any one of claims 29 to 32.

60. A method of modulating vascular permeability in a mammal, said method comprising administering to said mammal an effective vascular permeability modulating amount of a polypeptide according to any one of claims 16 to 24, or an antibody according to any one of claims 29 to 32.

61. A method according to claim 60, comprising administering to said mammal a polypeptide according to claim 16, having the ability to stimulate endothelial cell proliferation.

62. A method according to claim 60, comprising administering to said mammal a polypeptide according to claim 16, which has the ability to bind to endothelial cells, but which is unable to stimulate endothelial cell proliferation.

63. A method of activation of VEGF receptor 2, comprising the step of exposing cells bearing said receptor to an effective receptor activating dose of VEGF-D.

64. A method of activation of VEGF receptor 3, comprising the step of exposing cells bearing said receptor to an effective receptor activating dose of VEGF-D.

65. A method according to claim 63 or 64, wherein said method is carried out *in vivo*.

66. A method according to claim 63 or 64, wherein said method is carried out *in vitro*.

67. A diagnostic or prognostic test kit comprising a specific binding reagent for VEGF-D and means for detecting binding of said reagent.

68. A test kit according to claim 67, wherein said specific binding reagent comprises an antibody according to any one of claims 29 to 32.

69. A diagnostic or prognostic test kit comprising a pair of primers specific to VEGF-D DNA operatively coupled to a polymerase, whereby said polymerase is enabled to selectively amplify VEGF-D DNA from a DNA sample.

70. A method of detecting aberrations in VEGF-D gene structure in a test subject comprising the steps of:

providing a DNA sample from said test subject;

contacting said sample with a set of primers specific to VEGF-D DNA operatively coupled to a polymerase and selectively amplifying VEGF-D DNA from said sample by polymerase chain reaction; and

comparing the nucleotide sequence of the amplified VEGF-D DNA from said sample with a nucleotide sequence as set forth in SEQ ID NO:1 or SEQ ID NO:4.

71. A VEGF-D antagonist having the capability to inhibit at least one biological activity induced by VEGF-D selected from vascular permeability, endothelial cell proliferation and endothelial cell differentiation, said antagonist binding to VEGF-D or to a VEGF-D receptor, but being less able than VEGF-D to stimulate said at least one biological activity.

72. A VEGF-D antagonist according to claim 71, wherein said antagonist comprises an antibody which selectively binds VEGF-D.

73. A VEGF-D antagonist according to claim 72, wherein said antibody is a monoclonal antibody.

74. A VEGF-D antagonist according to claim 71, wherein said antagonist comprises a VEGF-D polypeptide fragment or analogue which binds to a VEGF-D receptor, but is less able to stimulate said at least one biological activity.

75. A method of improving pulmonary blood circulation and/or gas exchange in a mammal, said method comprising administering to said mammal an effective blood circulation and/or gas exchange improving amount of VEGF-D.

76. A method of treating fluid accumulation in the heart and/or lung due to increases in vascular permeability in a mammal, said method comprising administering to said mammal an effective vascular permeability decreasing amount of a VEGF-D antagonist.

77. A method of treating an intestinal malabsorption syndrome in a patient suffering therefrom, said method comprising administering to said patient an effective intestinal blood circulation and/or vascular permeability increasing amount of VEGF-D.

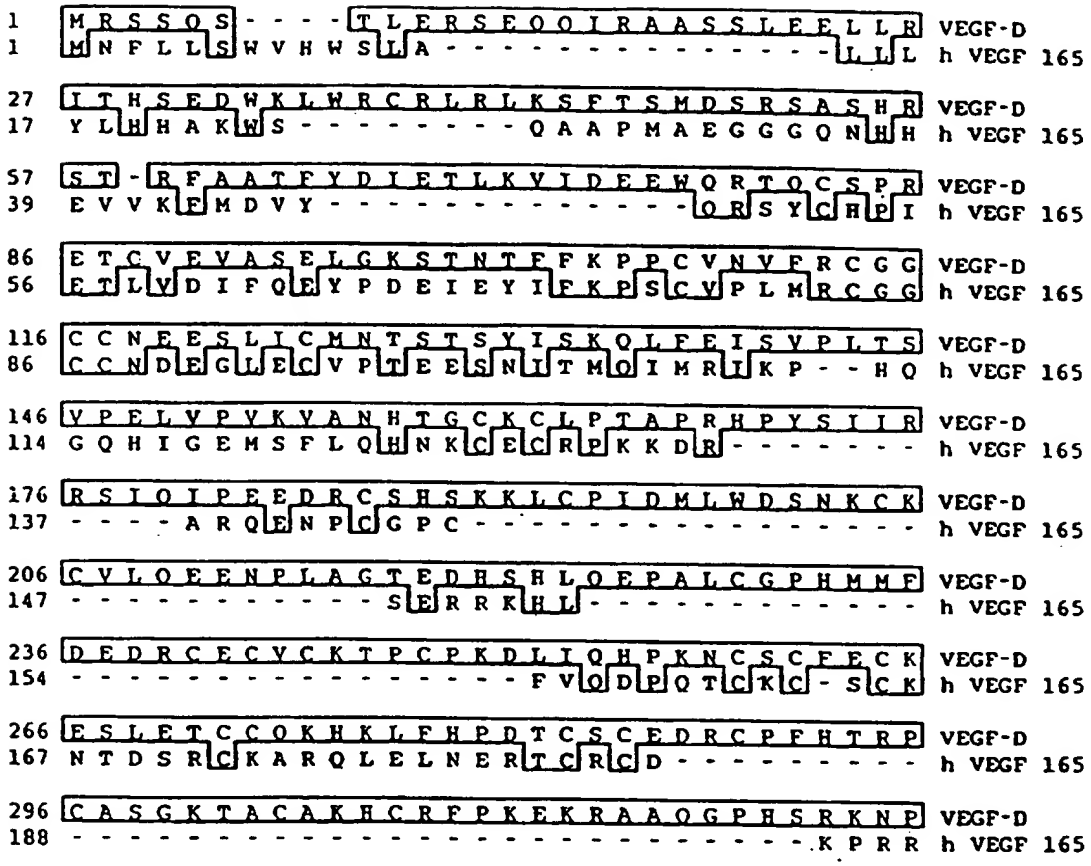
1 MRSSQS----TLERSEQQIRAASSLEELLR VEGF-D
1 MNFLLSWVHWSLA--------------LLLL h VEGF 165
27 ITHSEDWKLWRCRLRLKSFTSMDSRSASHR VEGF-D
17 YLHHAKWS--------QAAPMAEGGGQNHH h VEGF 165
57 ST-RFAATFYDIETLKVIDEEWQRTQCSPR VEGF-D
39 EVVKFMDVY--------------QRSYCHPI h VEGF 165
86 ETCVEVASELGKSTNTFFKPPCVNVFRCGG VEGF-D
56 ETLVDIFQEYPDEIEYIFKPSCVPLMRCGG h VEGF 165
116 CCNEESLICMNTSTSYISKQLFEISVPLTS VEGF-D
86 CCNDEGLECVPTEESNITMQIMRIKP--HQ h VEGF 165
146 VPELVPVKVANHTGCKCLPTAPRHPYSIIR VEGF-D
114 GQHIGEMSFLQHNKCECRPKKDR------- h VEGF 165
176 RSIQIPEDRCSHSKKLCPIDMLWDSNKCK VEGF-D
137 ----ARQENPCGPC---------------- h VEGF 165
206 CVLQEENPLAGTEDHSHLQEPALCGPHMMP VEGF-D
147 ----------SERRKHL------------ h VEGF 165
236 DEDRCECVCKTPCPKDLIQHPKNCSCFECK VEGF-D
154 ----------FVQDPQTCKC-SCK h VEGF 165
266 ESLETCCQKHKLFHPDTCSCEDRCPFHTRP VEGF-D
167 NTDSRCKARQLELNERTCRCD--------- h VEGF 165
296 CASGKTACAKHCRFPKEKRAAQGPHSRKNP VEGF-D
188 ----------------------KPRR h VEGF 165

FIG. 1a

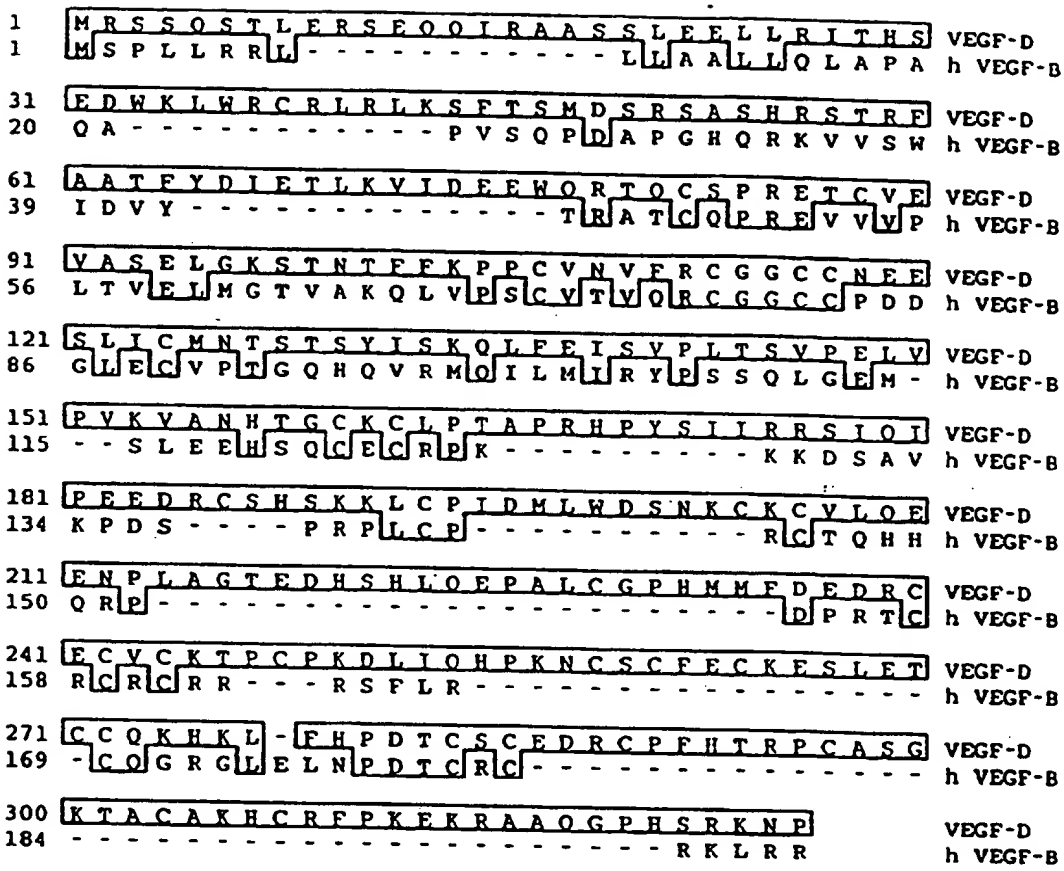
1 MRSSQSTLERSEQQIRAASSLEELLRITHS VEGF-D
1 MSPLLRRL----------LLAALLQLAPA h VEGF-B
31 EDWKLWRCRLRLKSFTSMDSRSASHRSTRF VEGF-D
20 QA----------PVSQPDAPGHQRKVVSW h VEGF-B
61 AATFYDIETLKVIDEEWQRTQCSPRETCVE VEGF-D
39 IDVY----------TRATCQPREVVVP h VEGF-B
91 VASELGKSTNTFFKPPCVNVFRCGGCCNEE VEGF-D
56 LTVEIMGTVAKQLVPSCVTVQRCGGCCPDD h VEGF-B
121 SLICMNTSTSYISKQLFEISVPLTSVPELV VEGF-D
86 GLECVPTGQHQVRMQILMIRYPSSQLGEM- h VEGF-B
151 PVKVANHTGCKCLPTAPRHPYSIIRRSIQI VEGF-D
115 --SLEEHSQCECRPK----------KKDSAV h VEGF-B
181 PEEDRCSHSKKLCPIDMLWDSNKCKCVLQE VEGF-D
134 KPDS----PRPLCP----------RCTQHH h VEGF-B
211 ENPLAGTEDHSHLQEPALCGPHMMFDEDRC VEGF-D
150 QRP----------DPRTC h VEGF-B
241 ECVCKTPCPKDLIQHPKNCSCFECKESLET VEGF-D
158 RCRCRR---RSFLR---------- h VEGF-B
271 CCQKHKL-FHPDTCSCEDRCPFHTRPCASG VEGF-D
169 -CQGRGLELNPDTCRC---------- h VEGF-B
300 KTACAKHCRFPKEKRAAQGPHSRKNP VEGF-D
184 ----------RKLRR h VEGF-B

FIG. 1b

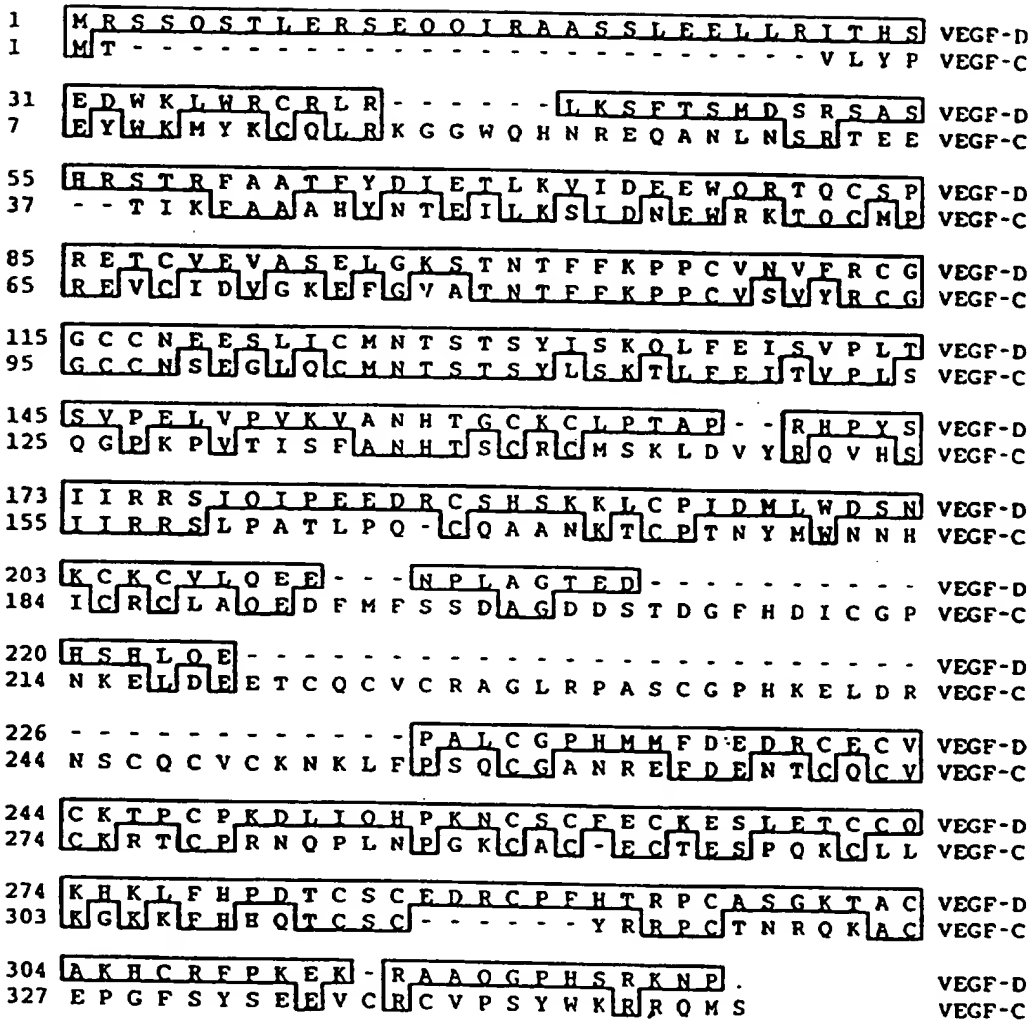
1 MRSSQSTLERSEQQIRAASSLEELLRITHS VEGF-D
1 MT-----------------------VLYP VEGF-C
31 EDWKLWRCRLR------LKSFTSMDSRSAS VEGF-D
7 EYWKMYKCQLRKGGWQHNREQANLNSRTEE VEGF-C
55 HRSTRFAATFYDIETLKVIDEEWQRTQCSP VEGF-D
37 --TIKFAAAHYNTEILKSIDNEWRKTQCMP VEGF-C
85 RETCVEVASELGKSTNTFFKPPCVNVFRCG VEGF-D
65 REVCIDVGKEFGVATNTFFKPPCVSVYRCG VEGF-C
115 GCCNEESLICMNTSTSYISKQLFEISVPLT VEGF-D
95 GCCNSEGLQCMNTSTSYLSKTLFEITVPLS VEGF-C
145 SVPELVPVKVANHTGCKCLPTAP--RHPYS VEGF-D
125 QGPKPVTISFANHTSCRCMSKLDVYRQVHS VEGF-C
173 IIRRSIQIPEEDRCSHSKKLCPIDMLWDSN VEGF-D
155 IIRRSLPATLPQ-CQAANKTCPTNYMWNNH VEGF-C
203 KCKCVLQEE---NPLAGTED---------- VEGF-D
184 ICRCLAQEDFMFSSDAGDDSTDGFHDICGP VEGF-C
220 HSHLQE---------------------- VEGF-D
214 NKELDEETCQCVCRAGLRPASCGPHKELDR VEGF-C
226 ----------PALCGPHMMFDEDRCECV VEGF-D
244 NSCQCVCKNKLFPSQCGANREFDENTCQCV VEGF-C
244 CKTPCPKDLIQHPKNCSCFECKESLETCCQ VEGF-D
274 CKRTCPRNQPLNPGKCACEECTESPQKCLL VEGF-C
274 KHKLFHPDTCSCEDRCPFHTRPCASGKTAC VEGF-D
303 KGKKFHHQTCSC------YRRPCTNRQKAC VEGF-C
304 AKHCRFPKEK-RAAQGPHSRKNP. VEGF-D
327 EPGFSYSEEVCRCVPSYWKRRQMS VEGF-C

FIG. 1c

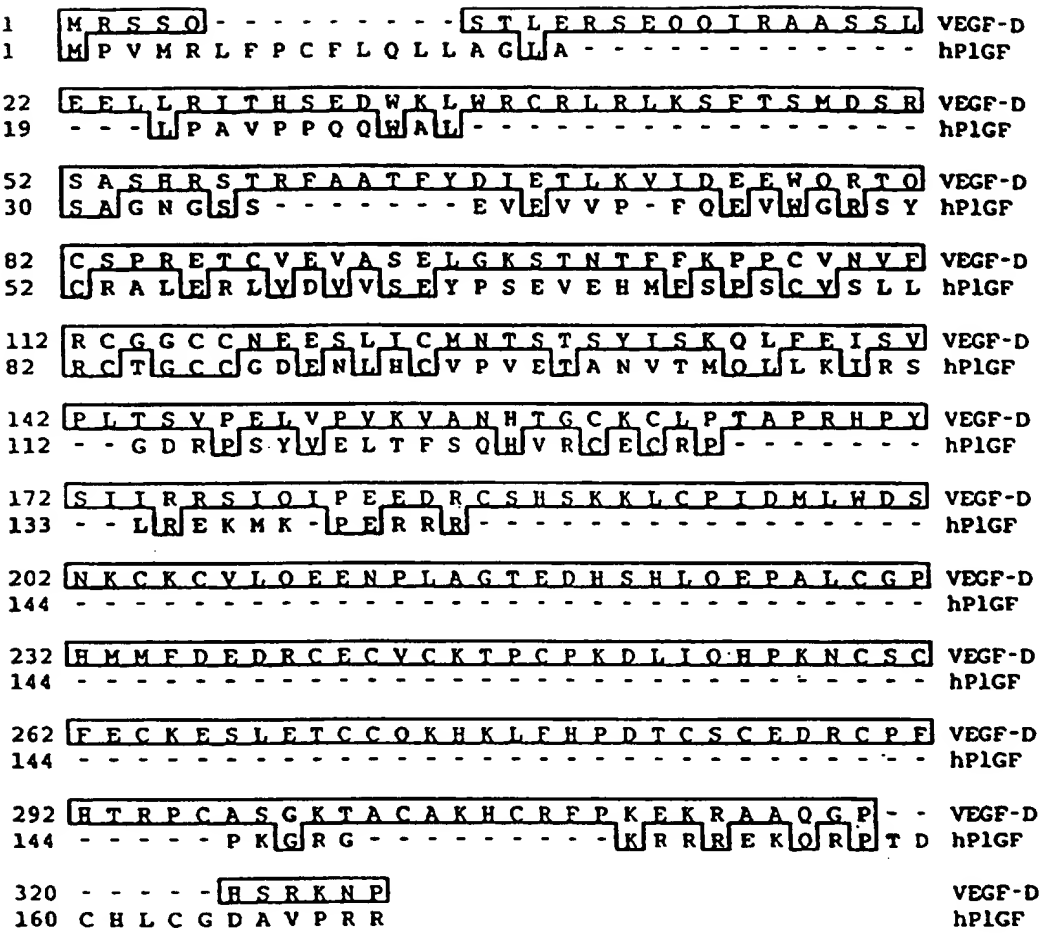
1 MRSSQ---------STLERSEQQIRAASSL VEGF-D
1 MPVMRLFPCFLQLLAGLA------------- hPlGF
22 EELLRITHSEDWKLWRCRLRLKSFTSMDSR VEGF-D
19 ---LPAVPPQQWALL---------------- hPlGF
52 SASHRSTRFAATFYDIETLKVIDEEWQRTQ VEGF-D
30 SAGNGSS-------EVEVVP-FQEVWGRSY hPlGF
82 CSPRETCVEVASELGKSTNTFFKPPCVNVF VEGF-D
52 CRALERLVDVVSEYPSEVEHMFSPSCVSLL hPlGF
112 RCGGCCNEESLICMNTSTSYISKQLFEISV VEGF-D
82 RCTGCCGDENLHCVPVETANVTMQLLKIRS hPlGF
142 PLTSVPELVPVKVANHTGCKCLPTAPRHPY VEGF-D
112 --GDRPSYVELTFSQHVRCECRP------- hPlGF
172 SIIRRSIQIPEEDRCSHSKKLCPIDMLWDS VEGF-D
133 --LREKMK-PERRR---------------- hPlGF
202 NKCKCVLQEENPLAGTEDHSHLQEPALCGP VEGF-D
144 ---------------------------- hPlGF
232 HMMFDEDRCECVCKTPCPKDLIQHPKNCSC VEGF-D
144 ---------------------------- hPlGF
262 FECKESLETCCQKHKLFHPDTCSCEDRCPF VEGF-D
144 ---------------------------- hPlGF
292 HTRPCASGKTACAKHCRFPKEKRAAQGP-- VEGF-D
144 -----PKGRG---------KRRRREKQRPTD hPlGF
320 -----HSRKNP VEGF-D
160 CHLCGDAVPRR hPlGF

FIG. 1d

FIG. 2

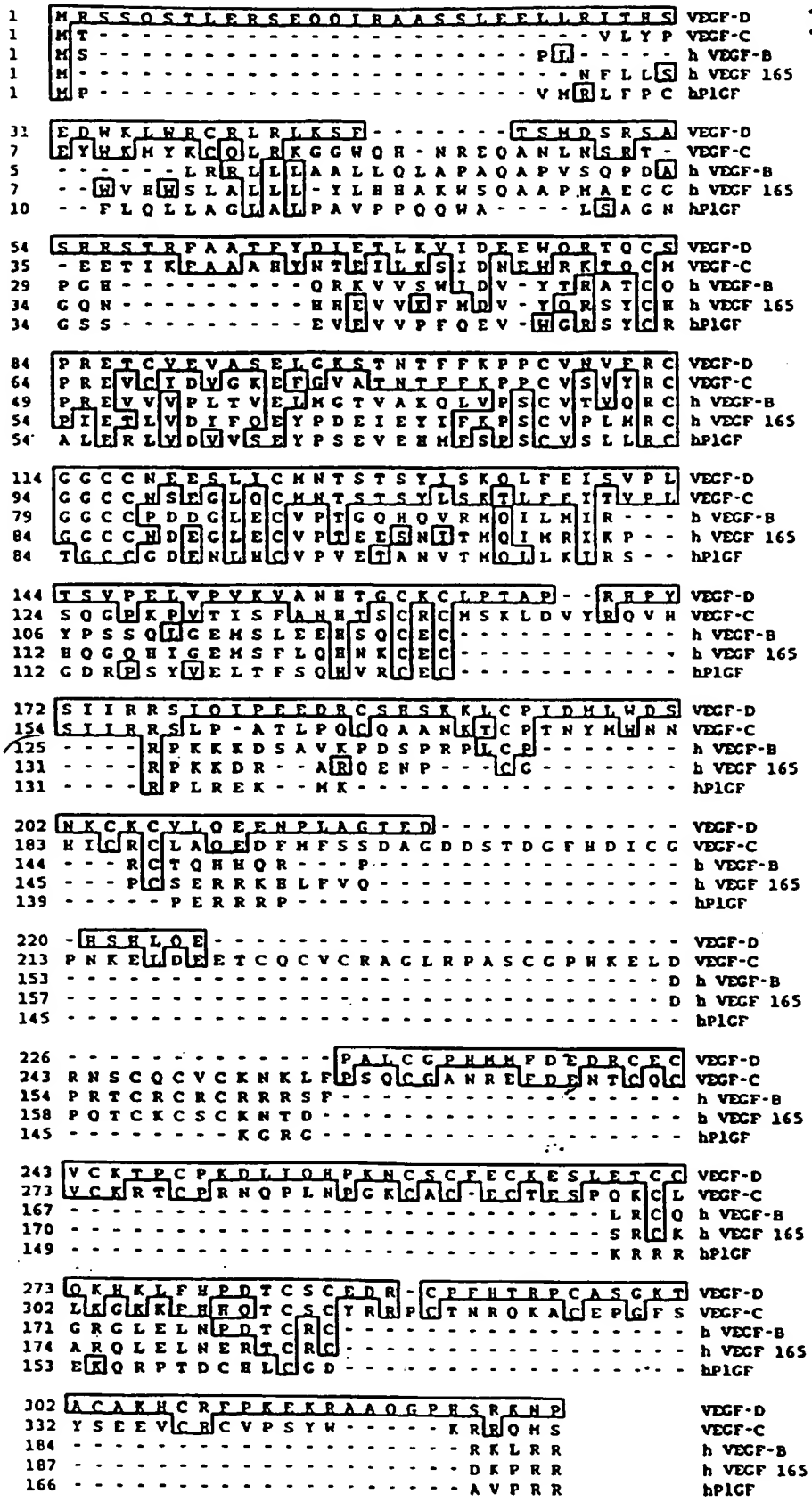

FIG. 3

```
                10                  20                  30                  40
MRSSQSTLERSEQQIRAASSLEELLRITHSEDWKLWRCRL

                50                  60                  70                  80
RLKSFTSMDSRSASHRSTRFAATFYDIETLKVIDEEWQRT

                90                  100                 110                 120
QCSPRETCVEVASELGKSTNTFFKPPCVNVFRCGGCCNEE

                130                 140                 150                 160
SLICMNTSTSYISKQLFEISVPLTSVPELVPVKVANHTGC

                170                 180                 190                 200
KCLPTAPRHPYSIIRRSIQIPEEDRCSHSKKLCPIDMLWD

                210                 220                 230                 240
SNKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRC

                250                 260                 270                 280
ECVCKTPCPKDLIQHPKNCSCFECKESLETCCQKHKLFHP

                290                 300                 310                 320
DTCSCEDRCPFHTRPCASGKTACAKHCRFPKEKRAAQGPH

SRKNP
```

FIG. 4

GTTGGGTTCCAGCTTTCTGTAGCTGTAAGCATTGGTGGCCACACCACCTCCTTACAA
AGCAACTAGAACCTGCGGCATACATTGGAGAGATTTTTTTAATTTTCTGGACATGAA
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MYREWVVVNVFMMLYVQLVQGSSNEHGPVKRSSQSTLERSEQQIRAASSLEELLRIT
HSEDWKLWRCRLRLKSFTSMDSRSASHRSTRFAATFYDIETLKVIDEEWQRTQCSPR
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LTSVPELVPVKVANHTGCKCLPTAPRHPYSIIRRSIQIPEEDRCSHSKKLCPIDMLW
DSNKCKCVLQEENPLAGTEDHSHLQEPALCGPHMMFDEDRCECVCKTPCPKDLIQHP
KNCSCFECKESLETCCQKHKLFHPDTCSCEDRCPFHTRPCASGKTACAKHCRFPKEK
RAAQGPHSRKNP

FIG. 5

FIG. 6

GGAGAATGCCTTTTGCAACACTTTTCAGTAGCTGCCTGGAAACAACTGCTTAGTCAT
CGGTAGACATTTAAAATATTCAAAATGTATGGAGAATGGGGAATGGGGAATATCCTC
ATGATGTTCCATGTGTACTTGGTGCAGGGCTTCAGGAGCGAACATGGACCAGTGAAG
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CGAGCAGCTTCTAGTTTGGAGGAGTTGCTGCAAATCGCGCACTCTGAGGACTGGAAG
CTGTGGCGATGCCGGTTGAAGCTCAAAAGTCTTGCCAGTATGGACTCACGCTCAGCA
TCCCATCGCTCCACCAGATTTGCGGCAACTTTCTATGACACTGAAACACTAAAAGTT
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GCCAGTGAGCTGGGGAAGACAACCAACACATTCTTCAAGCCCCCCTGTGTAAATGTC
TTCCGGTGTGGAGGCTGCTGCAACGAAGAGGGTGTGATGTGTATGAACACAAGCACC
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TTAGTGCCTGTTAAAATTGCCAACCATACGGGTTGTAAGTGCTTGCCCACGGGCCCC
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CCTCATTCCAAGAAACTCTGTCCTATTGACATGCTGTGGGATAACACCAAATGTAAA
TGTGTTTTGCAAGACGAGACTCCACTGCCTGGGACAGAAGACCACTCTTACCTCCAG
GAACCCACTCTCTGTGGACCGCACATGACGTTTGATGAAGATCGCTGTGAGTGCGTC
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GAGTGCAAAGAAAGTCTGGAGAGCTGCTGCCAAAAGCACAAGATTTTTCACCCAGAC
ACCTGCAGCTGTGAGGACAGATGTCCTTTTCACACCAGAACATGTGCAAGTAGAAAG
CCAGCCTGTGGAAAGCACTGGCGCTTTCCAAAGGAGACAAGGGCCCAGGGACTCTAC
AGCCAGGAGAACCCTTGATTCAACTTCCTTTCAAGTCCCCCCATCTCTGTCATTTTA
AACAGCTCACTGCTTTGTCAAGTTGCTGTCACTGTTGCCCACTACCCCTTGAACATG
TGCAAACACAGACACACACACACACACACACACAGAGCAACTAGAATTATGTTTTCT
AGGTGCTGCCTAAG

FIG. 7

AAACTTTGCTTCTGGAGAATGCCTTTTGCAACACTTTTCAGTAGCTGCCTGGAAACA
ACTGCTTAGTCATCGGTAGACATTTAAAATATTCAAAATGTATGGAGAATGGGGAAT
GGGGAATATCCTCATGATGTTCCATGTGTACTTGGTGCAGGGCTTCAGGAGCGAACA
TGGACCAGTGAAGCGATCATCCCGGTCCATGTTGGAACGATCTGAACAACAGATCCG
AGCAGCTTCTAGTTTGGAGGAGTTGCTGCAAATCGCGCACTCTGAGGACTGGAAGCT
GTGGCGATGCCGGTTGAAGCTCAAAAGTCTTGCCAGTATGGACTCACGCTCAGCATC
CGATCGCTCCACCAGATTTGCGGCAACTTTCTATGACACTGAAACACTAAAAGTTAT
AGATGAAGAATGGCAGAGGACCCAATGCAGCCCTAGAGAGACATGCGTAGAAGTCGC
CAGTGAGCTGGGGAAGACAACCAACACATTCTTCAAGCCCCCCTGTGTAAATGTCTT
CCGGTGTGGAGGCTGCTGCAACGAAGAGGGTGTGATGTGTATGAACACAAGCACCTC
CTACATCTCCAAACAGCTCTTTGAGATATCAGTGCCTCTGACATCAGTGCCCGAGTT
AGTGCCTGTTAAAATTGCCAACCATACGGGTTGTAAGTGCTTGCCCACGGGCCCCCG
CCATCCTTACTCAATTATCAGAAGATCCATTCAGACCCCAGAAGAAGATGAATGTCC
TCATTCCAAGAAACTCTGTCCTATTGACATGCTGTGGGATAACACCAAATGTAAATG
TGTTTTGCAAGACGAGACTCCACTGCCTGGGACAGAAGACCACTCTTACCTCCAGGA
ACCCACTCTCTGTGGACCGCACATGACGTTTGATGAAGATCGCTGTGAGTGCGTCTG
TAAAGCACCATGTCCGGGAGATCTCATTCAGCACCCGGAAAACTGCAGTTGCTTTGA
GTGCAAAGAAAGTCTGGAGAGCTGCTGCCAAAAGCACAAGATTTTTCACCCAGACAC
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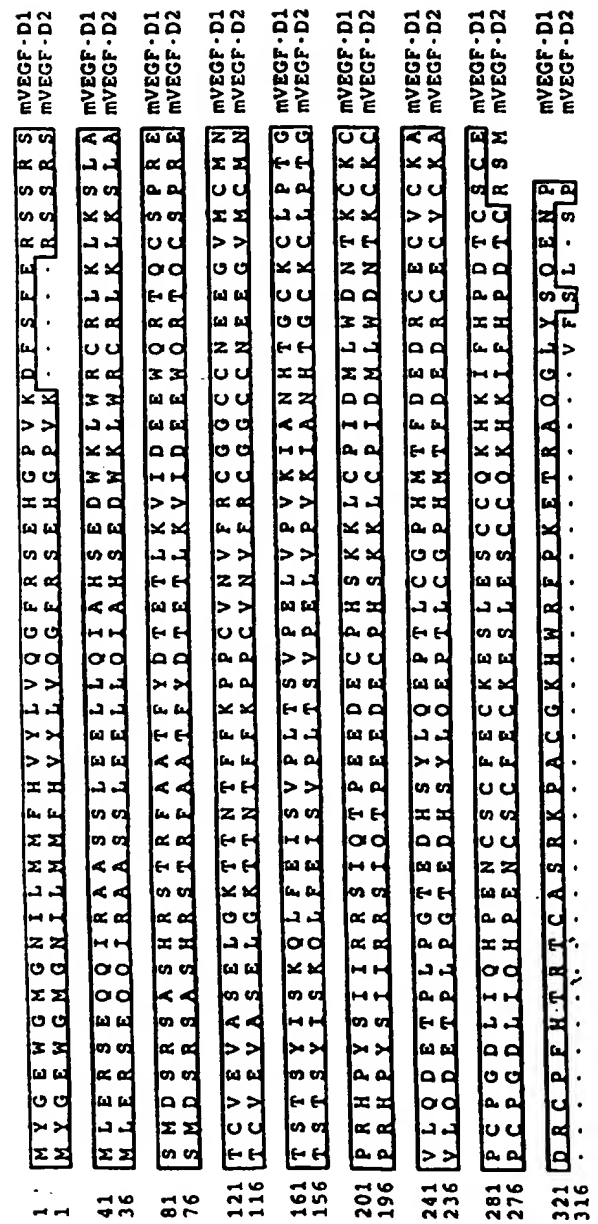

1 MYGEWGMGNILMMFHVYLVQGFRSEHGPVKDFSFERSSRS mVEGF-D1
1 MYGEWGMGNILMMFHVYLVQGFRSEHGPVK . . . . RSSRS mVEGF-D2
41 MLERSEQQIRAASSLEELLQIAHSEDWKLWRCRLKLKSLA mVEGF-D1
36 MLERSEQQIRAASSLEELLQIAHSEDWKLWRCRLKLKSLA mVEGF-D2
81 SMDSRSASHRSTRFAATFYDTETLKVIDEEWQRTQCSPRE mVEGF-D1
76 SMDSRSASHRSTRFAATFYDTETLKVIDEEWQRTQCSPRE mVEGF-D2
121 TCVEVASELGKTTNTFFKPPCVNVFRCGGCCNEEGVMCMN mVEGF-D1
116 TCVEVASELGKTTNTFFKPPCVNVFRCGGCCNEEGVMCMN mVEGF-D2
161 TSTSYISKQLFEISVPLTSVPELVPVKIANHTGCKCLPTG mVEGF-D1
156 TSTSYISKQLFEISVPLTSVPELVPVKIANHTGCKCLPTG mVEGF-D2
201 PRHPYSIIRRSIQTPEEDECPHSKKLCPIDMLWDNTKCKC mVEGF-D1
196 PRHPYSIIRRSIQTPEEDECPHSKKLCPIDMLWDNTKCKC mVEGF-D2
241 VLQDETPLPGTEDHSYLQEPTLCGPHMTFDEDRCECVCKA mVEGF-D1
236 VLQDETPLPGTEDHSYLQEPTLCGPHMTFDEDRCECVCKA mVEGF-D2
281 PCPGDLIQHPENCSCFECKESLESCCQKHKIFHPDTCSCE mVEGF-D1
276 PCPGDLIQHPENCSCFECKESLESCCQKHKIFHPDTCRSM mVEGF-D2
321 DRCPFHTRTCASRKPACGKHWRFPKETRAQGLYSQENP mVEGF-D1
316 . . . . . VFSLSP mVEGF-D2

FIG. 8

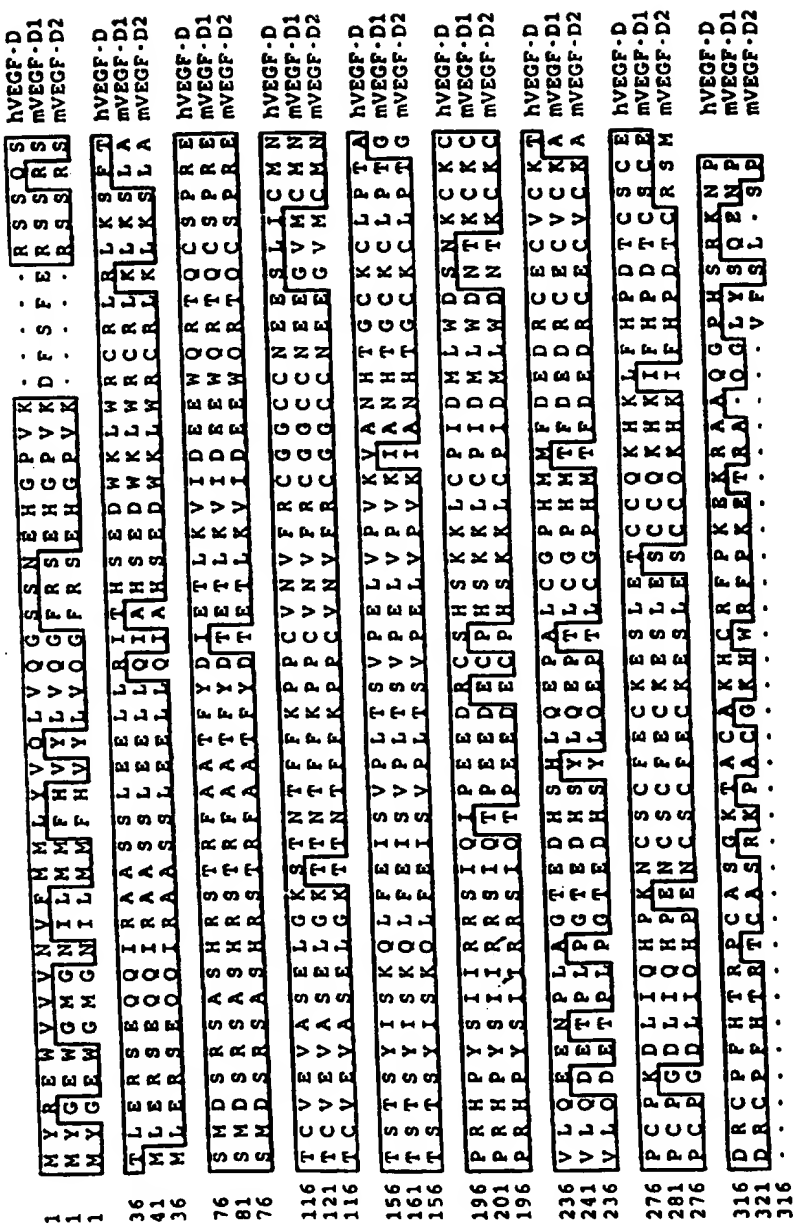


FIG. 9

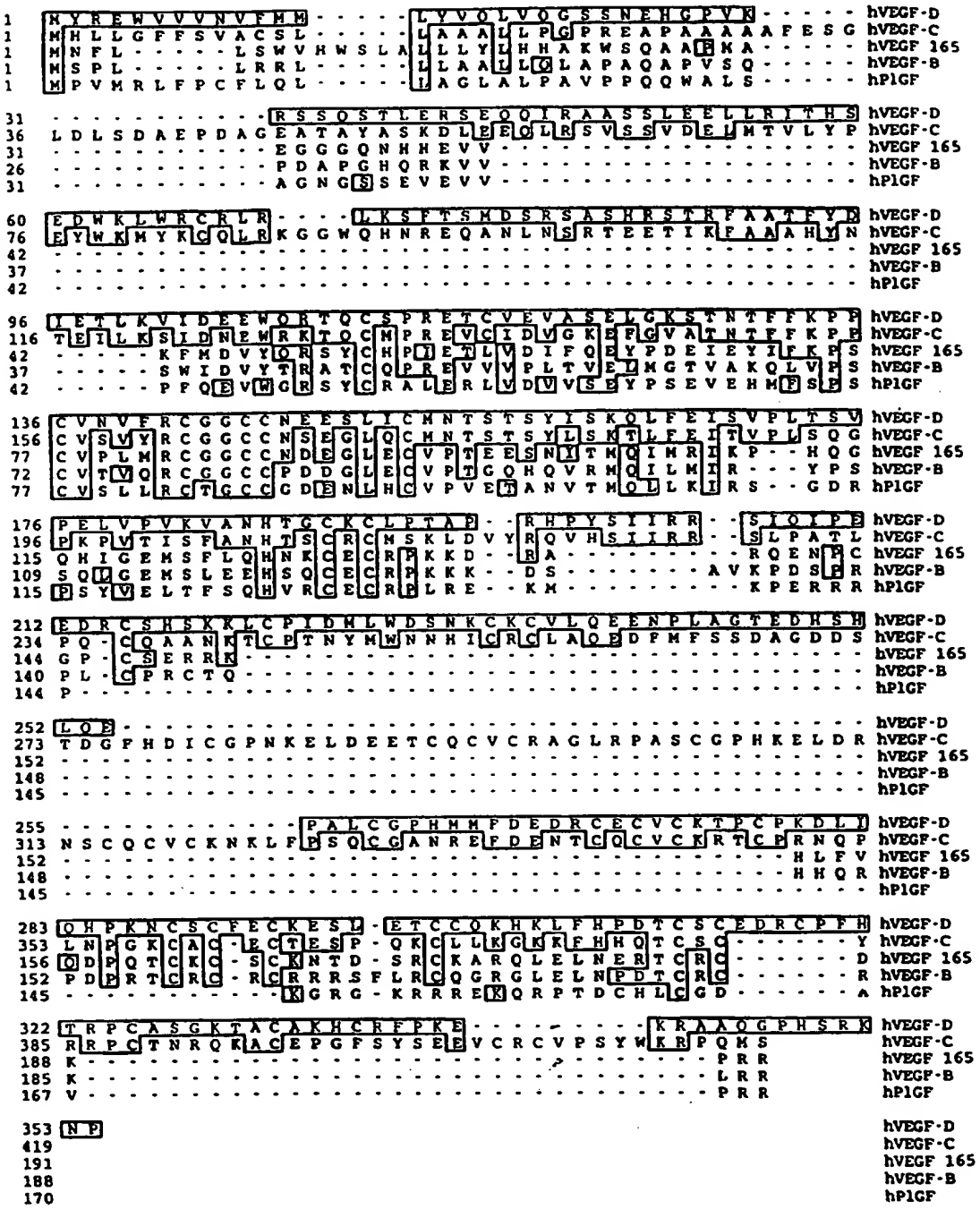
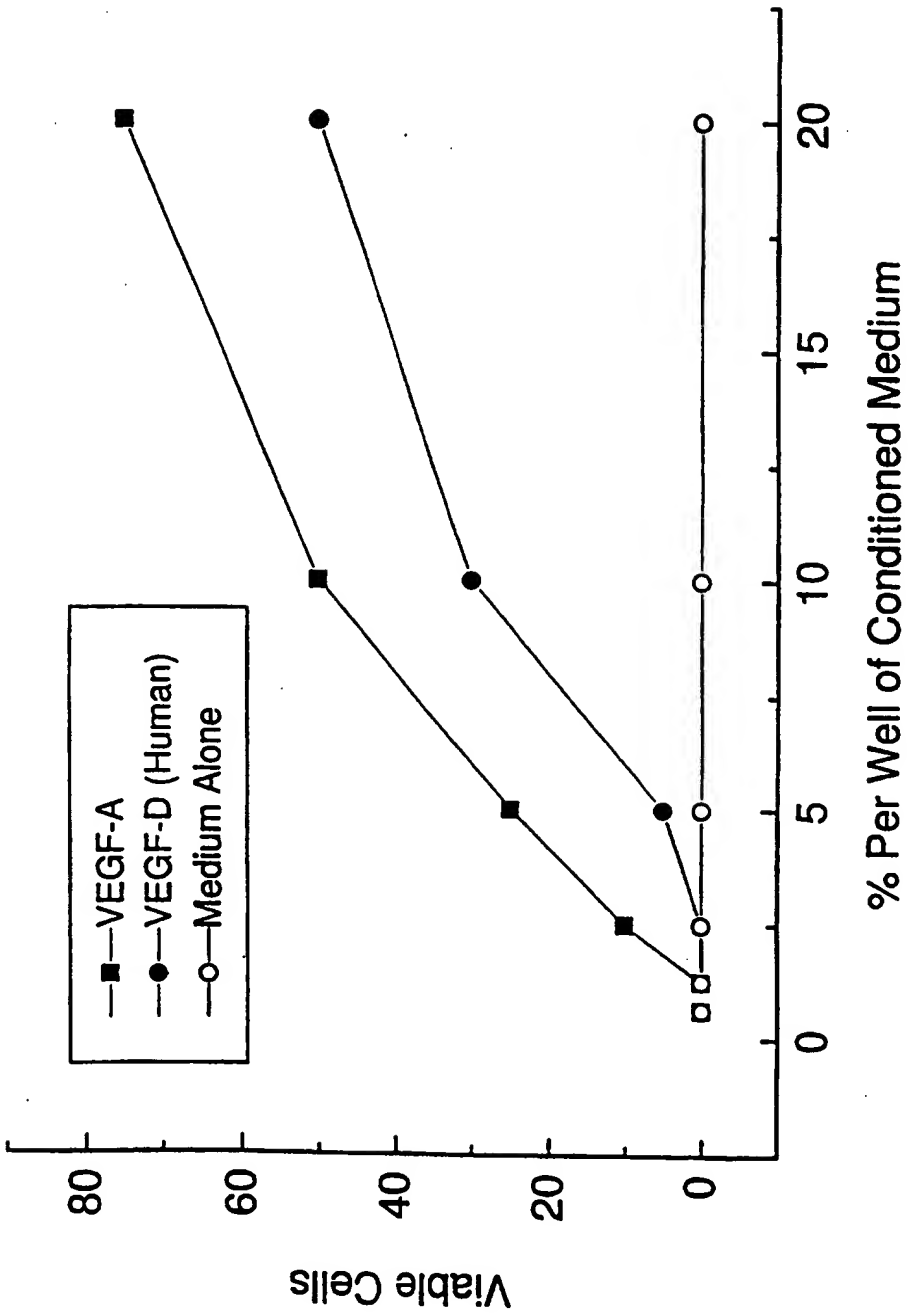


FIG. 10

FIG. 11 VEGFR2 bioassay. Testing of COS cell conditioned medium containing VEGF-A, VEGF-D(Human)



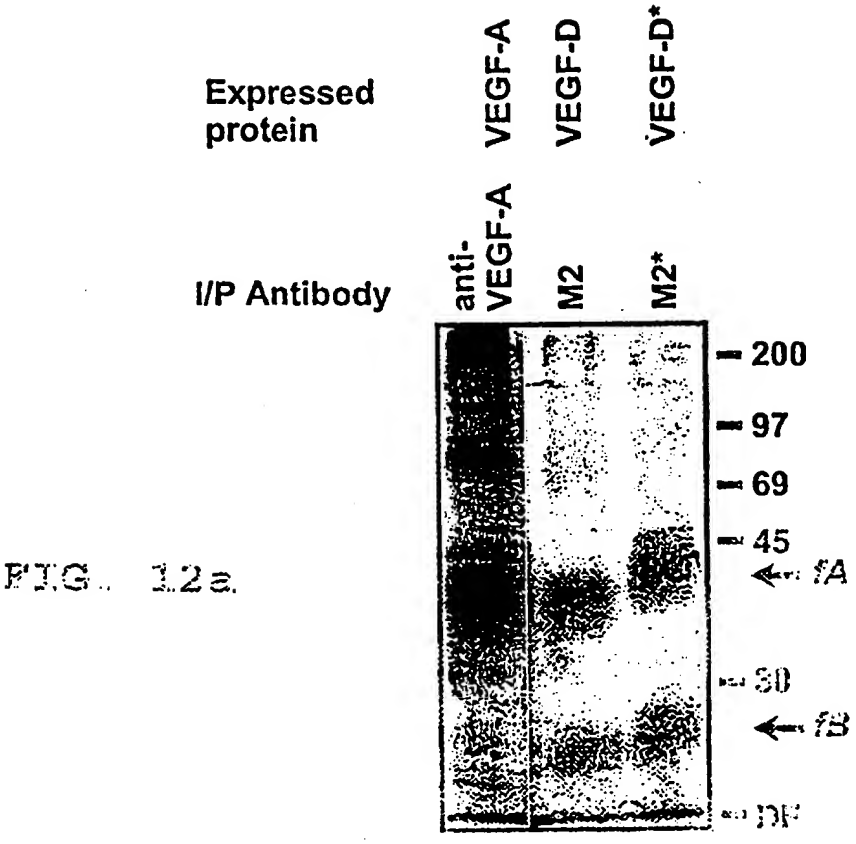


FIG. 12a

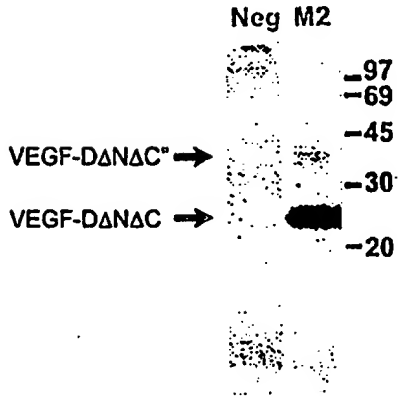
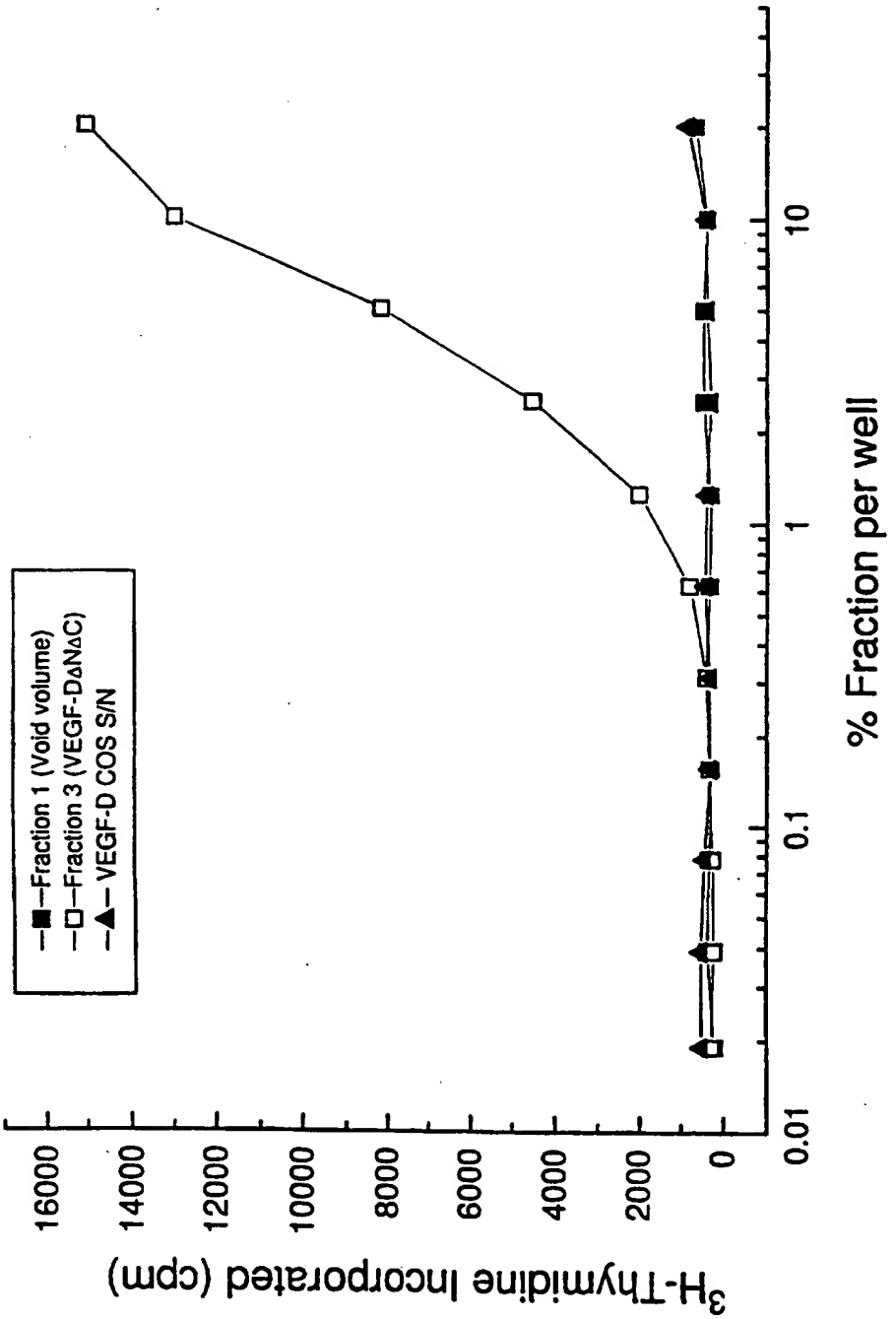


FIG. 12b

FIG. 13


3H-Thymidine Incorporated (cpm)
16000
14000
12000
10000
8000
6000
4000
2000
0
0.01
0.1
1
10
% Fraction per well
—■—Fraction 1 (Void volume)
—□—Fraction 3 (VEGF-DΔNΔC)
—▲— VEGF-D COS S/N

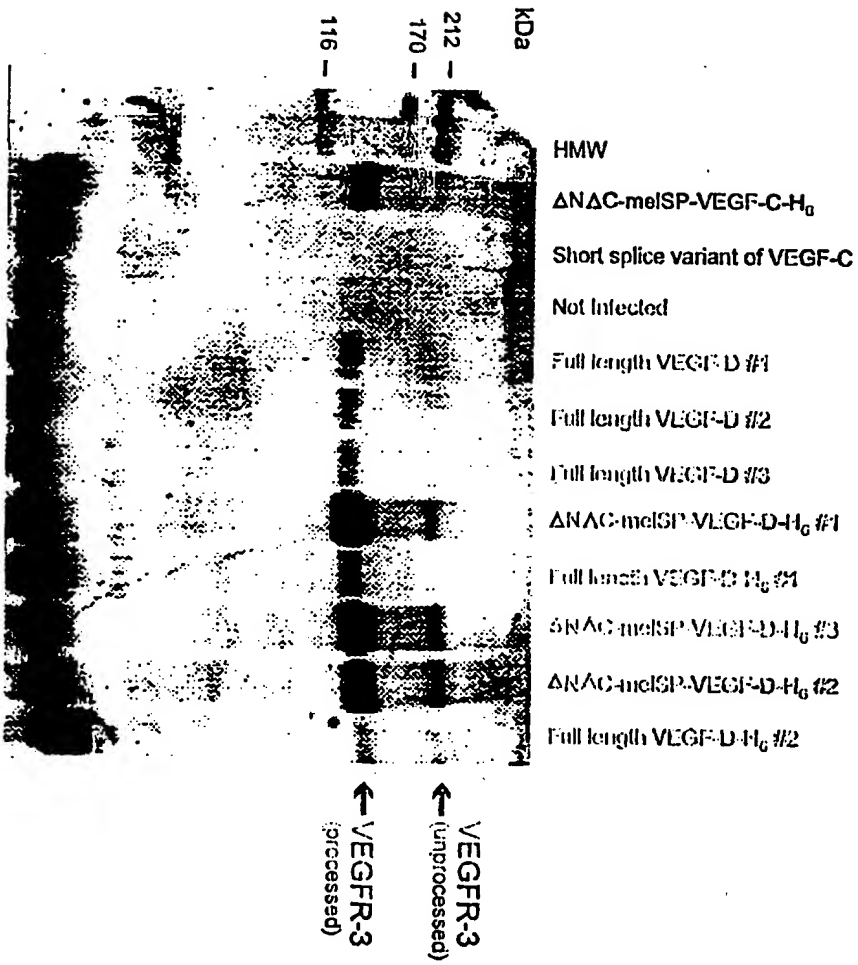

kDa
212 –
170 –
116 –
HMW
ΔNΔC-melSP-VEGF-C-Hc
Short splice variant of VEGF-C
Not Infected
Full length VEGF-D #1
Full length VEGF-D #2
Full length VEGF-D #3
ΔNΔC-melSP-VEGF-D-Hc #1
Full length VEGF-D Hc #1
ΔNΔC-melSP-VEGF-D-Hc #3
ΔNΔC-melSP-VEGF-D-Hc #2
Full length VEGF-D-Hc #2
VEGFR-3 (processed)
VEGFR-3 (unprocessed)

FIG. 14

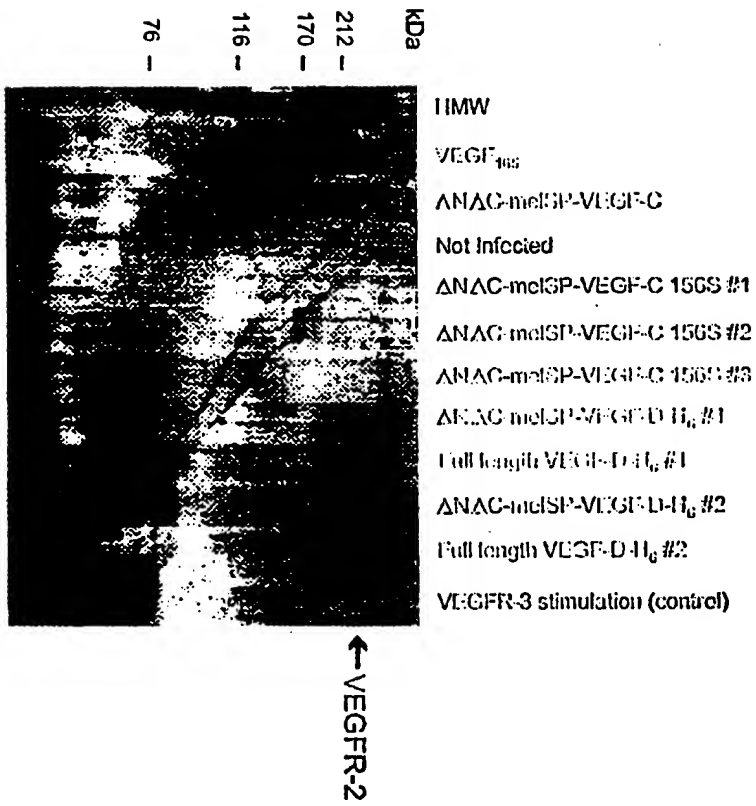
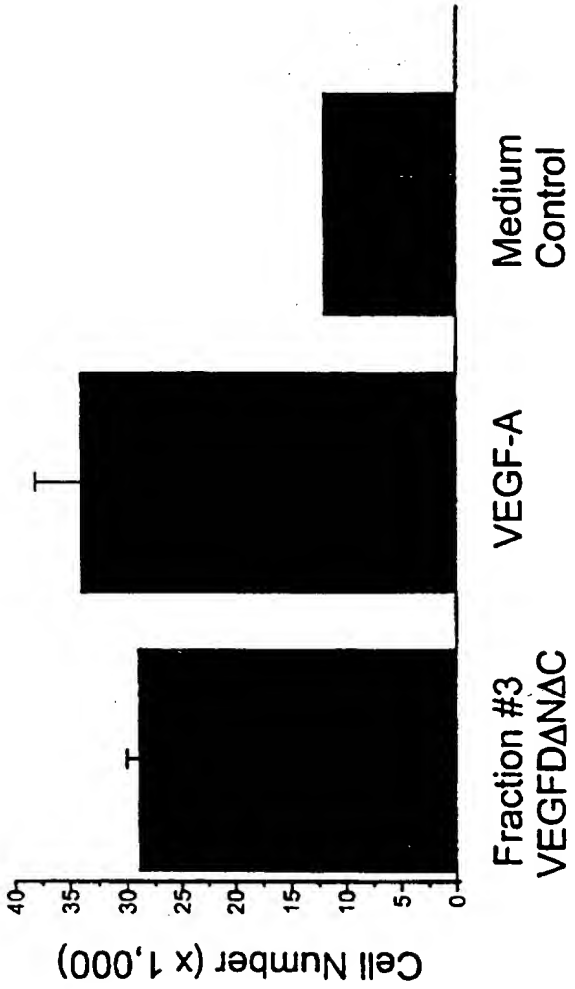

kDa
212 –
170 –
116 –
76 –
HMW
VEGF165
ΔNAC-melGP-VEGF-C
Not Infected
ΔNAC-melGP-VEGF-C 156S #1
ΔNAC-melGP-VEGF-C 156S #2
ΔNAC-melGP-VEGF-C 156S #3
ΔNAC melGP-VEGF-D Hc #1
Full length VEGF-D Hc #1
ΔNAC-melSP-VEGF-D-Hc #2
Full length VEGF-D Hc #2
VEGFR-3 stimulation (control)
VEGFR-2

FIG. 15

FIG. 16


Cell Number (x 1,000)
0
5
10
15
20
25
30
35
40
Fraction #3 VEGFDΔNΔC
VEGF-A
Medium Control

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14696

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 1/21, 5/10, 15/18, 15/63; C07K 1/14, 14/475, 14/49

US CL :435/69.4, 325, 243, 320.1; 530/399, 412; 536/23.51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.4, 325, 243, 320.1; 530/399, 412; 536/23.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database GenBank, Accession Number H24828, HILLIER et al. y142g02.r1 Homo sapiens cDNA clone 160946 5', 07 July 1995, see entire document.	6
X,P	ORLANDINI et al. Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc. Natl. Acad. Sci. USA. October 1996, Vol. 93, pages 11675-11680, see especially Figure 1 and "Materials and Methods" at pages 11675-11677.	1-3, 5-6, 8, 16-23, 33, 35
A	TISCHER et al. Vascular endothelial growth factor: a new member of the platelet-derived growth factor gene family. Biochm. Biophys. Res. Comm. Decemer 1989, Vol. 165, No. 3, pages 1198-1206, see entire document.	1-13, 16-28, 33-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search . 29 OCTOBER 1997	Date of mailing of the international search report 23 DEC 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHRISTINE SAOUD Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14696

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 14-15, 29-32, 51-57, 59-62, 68
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-13, 16-28, 33-35

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REP RT

International application No.
PCT/US97/14696

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, BIOSIS, MEDLINE, EMBASE, WPIDS
search terms: vascular endothelial cell growth factor, VEGF, PDGF, VEGF-D, endothelial cell proliferation, vascular permeability

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-13, 16-28, 33-35, drawn to DNA, vectors, host cells, polypeptides, pharmaceutical compositions of polypeptides and methods of making polypeptides.

Group II, claim(s) 36, drawn to a vector comprising antisense molecule.

Group III, claim(s) 37-45, 63-66 (in part), drawn to methods of promoting cell differentiation and/or proliferation.

Group IV, claim(s) 39-40, 63-66 (in part), drawn to methods of stimulating vascular permeability.

Group V, claim(s) 46-49, drawn to methods of inhibiting by administration of an antagonist which is an antibody.

Group VI, claim(s) 50, drawn to a method of inhibiting expression by administration of an antisense vector.

Group VII, claim(s) 58, drawn to methods of detecting VEGF-D.

Group VIII, claim(s) 67 (in part), drawn to a diagnostic test kit which uses an antibody.

Group IX, claim(s) 67 (in part), 69, drawn to a diagnostic test kit which uses primers.

Group X, claim(s) 70, drawn to methods of detecting aberrations in VEGF-D.

Group XI, claim(s) 71 (in part), 72, 73, drawn to a VEGF-D antagonist which is an antibody and pharmaceutical compositions thereof.

Group XII, claim(s) 71 (in part), 74, drawn to a VEGF-D antagonist which is a fragment or analog of VEGF-D.

Group XIII, claim(s) 76, drawn to methods of using a VEGF-D antagonist.

Group XIV, claim(s) 75, drawn to a method of increasing circulation and/or gas exchange.

Group XV, claim(s) 77, drawn to a method of treating intestinal malabsorption.

The inventions listed as Groups I-XV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of Group I, DNA encoding a VEGF polypeptide, is not shared by all of inventive groups. Therefore, the main inventive groups is the DNA encoding a VEGF polypeptide, including the vectors and host cells containing said DNA, the polypeptide made with said DNA and the first method of using the DNA, which is the method of making the protein. The special technical feature of Group II is the vector containing the antisense. The special technical feature of Group III is the method of promoting cell differentiation and/or proliferation. The special technical feature of Group IV is the method of stimulating vascular permeability. The special technical feature of Group V is the method of inhibition by administration of an antibody. The special technical feature of Group VI is the method of inhibiting expression by administration of an antisense vector. The special technical feature of Group VII is the method of detecting VEGF-D. The special technical feature of Group VIII is the diagnostic test kit which utilizes an antibody. The special technical feature of Group IX is the diagnostic test kit which utilizes primers. The special technical feature of Group X is the method of detecting aberrations in VEGF-D. The special technical feature of Group XI is the VEGF-

D antibody. The special technical feature of Group XII is the VEGF-D antagonist which is an analog or fragment of VEGF-D. The special technical feature of Group XIII is the method of using an antagonist. The special technical feature of Group XIV is the method of increasing circulation and/or gas exchange. The special technical feature of Group XV is the method of treating intestinal malabsorption.

PCT Rule 13 does not provide for multiple products or methods within a single application. The methods are distinct because they have different goals, method steps, and/or starting materials as well as being qualitatively different methods. The products are distinct because they do not share the same or corresponding technical feature (i.e. DNA encoding VEGF-D) and because they are structurally different products.

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 11709–11714, September 1998
Cell Biology

Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells

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*Ludwig Institute for Cancer Research, Stockholm Branch, Box 240, S-171 77 Stockholm, Sweden; ‡Molecular/Cancer Biology Laboratory, Haartman Institute, P.O. Box 21 (Haartmaninkatu 3), 00014 Helsinki, Finland; §Department of Morphology, University Medical Center, 1211 Geneva 4, Switzerland; and ¶Institute for Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan

Edited by M. Judah Folkman, Harvard Medical School, Boston, MA, and approved August 10, 1998 (received for review January 9, 1998)

ABSTRACT **The vascular endothelial growth factor (VEGF) family has recently expanded by the identification and cloning of three additional members, namely VEGF-B, VEGF-C, and VEGF-D. In this study we demonstrate that VEGF-B binds selectively to VEGF receptor-1/Flt-1. This binding can be blocked by excess VEGF, indicating that the interaction sites on the receptor are at least partially overlapping. Mutating the putative VEGF receptor-1/Flt-1 binding determinants Asp^{63}, Asp^{64}, and Glu^{67} to alanine residues in VEGF-B reduced the affinity to VEGF receptor-1 but did not abolish binding. Mutational analysis of conserved cysteines contributing to VEGF-B dimer formation suggest a structural conservation with VEGF and platelet-derived growth factor. Proteolytic processing of the 60-kDa VEGF-B_{186} dimer results in a 34-kDa dimer containing the receptor-binding epitopes. The binding of VEGF-B to its receptor on endothelial cells leads to increased expression and activity of urokinase type plasminogen activator and plasminogen activator inhibitor 1, suggesting a role for VEGF-B in the regulation of extracellular matrix degradation, cell adhesion, and migration.**

Vascular endothelial growth factor (VEGF) has been implicated as a key regulator of blood vessel formation (1). It is required for both vasculogenesis, where mesoderm-derived angioblasts form tubes, and for angiogenesis, where capillaries form by sprouting or intussusception from existing vessels (2). While vasculogenesis is restricted to embryonic development, angiogenesis continues to operate throughout life when neovascularization is required. Physiological angiogenesis is mainly restricted to the female reproductive cycle and wound healing, but the angiogenic machinery can also be recruited by pathological processes such as tumor growth (3).

VEGF exerts its functions through binding to two receptor tyrosine kinases, VEGFR-1/Flt-1 and VEGFR-2/KDR (1). These receptors are expressed almost exclusively on endothelial cells, although VEGFR-1 is also found in monocytes, where it mediates migration (4, 5). Targeted homozygous null mutations of both receptor genes result in arrest of embryonic development (6, 7). Disruption of the VEGFR-1 gene interferes with the organization of the vascular endothelium (6), whereas VEGFR-2 is required for endothelial cell differentiation and definitive hematopoiesis (7, 8). VEGF levels are critical for normal development, as inactivation of even one allele results in embryonic death (9, 10).

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VEGF has been shown to regulate most steps of the angiogenic process, including endothelial cell degradation of extracellular matrix (ECM), migration, proliferation, and tube formation (1). In keeping with its ability to induce ECM degradation, VEGF increases the expression and activity of plasminogen activators, urokinase type plasminogen activator (uPA) and tissue type plasminogen activator (tPA) (11). These serine proteases convert plasminogen to plasmin and are thereby involved in tissue remodeling, cell invasion, and thrombolysis (reviewed in ref. 12). Whereas tPA is a fibrin-dependent intravascular enzyme, uPA functions as a receptor (uPAR)-bound cell surface activator. Both proteases are specifically inhibited by plasminogen activator inhibitor type 1 (PAI-1), the expression of which is also up-regulated by VEGF (11). This inhibition may serve to protect ECM from excessive proteolysis, as concerted expression of PAI-1 and uPA has been observed during physiological angiogenesis *in vivo* (13). Interestingly, both PAI-1 and uPA/uPAR have recently been implicated in regulation of cell adhesion and migration (12). uPAR and PAI-1 compete for binding to vitronectin, and PAI-1 regulates adhesion also directly by competing with integrin $\alpha_v\beta_3$ for vitronectin binding. Taken together, the uPA/uPAR/PAI-1 system may have a dual role: it can regulate proteolysis and cellular adhesiveness, the latter being independent of the enzymatic function.

The VEGF family of growth factors comprises at present five members—i.e., VEGF, placenta growth factor (PlGF) (14), VEGF-B/VRF (15, 16), VEGF-C/VRP (17, 18), and VEGF-D/FIGF (19, 20). While PlGF binds selectively to VEGFR-1 (21), VEGF-C and VEGF-D bind both VEGFR-3/Flt-4 and VEGFR-2 (17, 22). The corresponding receptor(s) for VEGF-B has not been reported. VEGF-B resembles PlGF in two aspects: it exists as two alternatively spliced forms, VEGF-B_{167} and VEGF-B_{186}, which differ in their affinity for heparin and thus release and bioavailability, and it forms heterodimers with VEGF (15, 23), a property likely to alter its receptor specificity and biological effects. In contrast to PlGF, however, VEGF-B is widely expressed and is most prominent in heart and skeletal muscle (15).

Alanine-scanning mutagenesis of VEGF has implicated the negatively charged amino acid residues Asp^{63}, Glu^{64}, and Glu^{67} in VEGFR-1 binding (24). These acidic amino acid residues are conserved in VEGF-B and to lesser extent in PlGF.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: ECM, extracellular matrix; PAI-1, plasminogen activator inhibitor type 1; PlGF, placenta growth factor; tPA, tissue type plasminogen activator; uPA, urokinase type plasminogen activator; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; h-, human; m-, mouse; bFGF, basic fibroblast growth factor.
†B.O. and E.K. contributed equally to this work, as did K.A. and U.E.
‖To whom reprint requests should be addressed. e-mail: ueri@licr.ki.se or Kari.Alitalo@helsinki.fi.

Covalent dimerization of VEGF has been shown to be required for its biological activity (25), and recently the determination of the crystal structure verified the antiparallel arrangement of the two subunits covalently linked by two disulfide bridges between Cys^{51} and Cys^{60} (26). The eight conserved cysteine residues characteristic of the PDGF/VEGF growth factor family imply structural conservation between the members (for recent sequence alignment see ref. 20).

In this work we report that VEGF-B binds specifically to VEGFR-1. Mutation of the putative receptor-binding determinants to alanine residues reduced the affinity to VEGFR-1 but did not abolish receptor binding, and mutations in conserved cysteine residues predict that VEGF-B forms antiparallel dimers. Furthermore, we show that VEGF-B_{186} is proteolytically processed, and we analyze the ability of VEGF-B to regulate the uPA/PAI-1 system in endothelial cells.

MATERIALS AND METHODS

Cell Culture and Materials. Sf9 cells were maintained in Sf-900 II SFM (GIBCO/BRL Life Technologies) supplemented with 0.1% pluronic f-68 for suspension growth, High Five cells (Invitrogen) in Ex-cell 400 medium (JHR Bioscience; Lenexa, KS), and Schneider 2 (S2) cells (Invitrogen) were grown in DES-medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). 293-EBNA, 293-T, and NIH 3T3-Flt1 cells (27) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. NIH 3T3-Flt1 cells were kept under continuous selection with 200 μg/ml neomycin. Bovine adrenal cortex-derived microvascular endothelial (BME) cells were kindly provided by M. B. Furie and S. C. Silverstein (28) and were grown in MEMα (GIBCO; Basel, Switzerland) supplemented with 15% donor calf serum on 1.5% gelatin-coated tissue culture flasks. Plasmin was purchased from Boehringer Mannheim, anti-VEGF (MAB 293) from R & D Systems, and human (h)$VEGF_{165}$ from R & D Systems or from Preprotech (Rocky Hill, NJ). Recombinant mouse (m)$VEGF_{164}$ and human basic fibroblast growth factor (bFGF) (155 amino acid form) were kind gifts from H. Weich (GBF, Braunschweig Germany) and P. Sarmientos (Farmitalia Carlo Erba, Milan), respectively.

Construction of Expression Plasmids and Generation of VEGF-B Mutants. The expression plasmid pIg-VEGFR-1 coding for the first five Ig-like domains of VEGFR-1 fused to human IgG1 Fc was constructed by ligating a *Hin*dIII fragment (coding for amino acids 1–549 of VEGFR-1) from pLTR-Flt1 into pIgplus vector (Ingenius; Novagen). Prior to the cloning the latter had been digested with *Xho*I and *Xba*I, blunted, and religated to correct the reading frame for the fusion protein production. For the spIg-VEGFR-2 construct, cDNA encoding the first four Ig-like domains of VEGFR-2 was amplified by PCR using human fetal lung cDNA library (CLONTECH) as a template. The primers 5′-ATGGTACCCCCAGGCTCAGCATACAAAAAGAC-3′ and 5′-GCGTCTAGAGGGTGGGACATACACAACCAG-3′were used and the amplified fragment was cleaved with *Kpn*I and *Xba*I and inserted into corresponding sites of signal pIg vector (Ingenius). mVEGF-B_{186} cDNA (23) was cleaved by *Eco*RI and subcloned in pFASTBAC1 (GIBCO/BRL Life Technologies). A $(His)_6$ tag and an enterokinase site were introduced at the N terminus, devoid of signal sequence, using PCR with mVEGF-B_{186} pSG5 as a template and the primers 5′-ATCGAGATCTTCATCACCATCACCATCACGGAGATGACGATGACAAACCTGTGTCCCAGTTT-3′ and 5′-CAAGGGCGGGGCTTAGAGATCTAGCT-3′ (both containing *Bgl*II sites). The amplified fragment was cleaved with *Bgl*II and inserted into the *Bam*HI site, in frame with the signal sequence of gp67 of pAcGP67A (PharMingen). Human VEGF-B_{186} cDNA was amplified by PCR using the forward primer 5′-GGAATTCCCCGCCCAGGCCCCTGTC-3′ and the reverse primer 5′-GGAATTCAATGATGATGATGATGAGCCCCGCCCTTGGC-3′. The amplified product containing a C-terminal $(His)_6$ tag was inserted into the *Eco*RI site of pPIC-9 (Invitrogen), in frame with the α mating factor signal sequence. The cysteine-to-serine mutants and the alanine mutants in mVEGF-B_{167} pSG5 as well as mVEGF-B exon 1–5 mutant containing a C-terminal Kemptide motif (29), VEGF-B_{kEx1-5} pSG5, were generated by M13-based *in vitro* single-stranded mutagenesis employing the helper phage M13KO7 (30) and the *dut*$^-$ *ung*$^-$ *Escherichia coli* strain RZ1032 (31). The primers 5′-ACGTAGATCTCCTGTGTCCCAG-3′ and 5′-ACGTGAATTCTCAGCTGTCTGGCTTCAC -3′ (introducing a stop codon after exon 5) were used to PCR-amplify the alanine mutants, which were subcloned in pMT/Bip/V5-HisC (Invitrogen). All constructs were verified by sequencing.

Protein Expression and Purification. For production of recombinant baculoviral protein in Sf9 and High Five cells, mVEGF-B recombinant plaques were purified and amplified (32), and the corresponding expressed proteins as well as hVEGF-B_{186} expressed in *Pichia pastoris* (strain GS115) were purified by using Ni-NTA Superflow resin (Qiagen). For ligand competition assay, High Five cells were infected with mVEGF-B_{186} pFASTBAC1 virus or with a mock virus, and the media were harvested 48 hr after infection and immediately used or frozen at −70°C. The S2 cells were transfected and the expression was induced according to the supplier. The conditioned media were collected 72 hr after induction.

Antibodies. Purified m$(His)_6$VEGF-B_{186} protein was used for immunization of rabbits according to standard procedures. The obtained antiserum and the antiserum to mVEGF-B N-terminal peptide (23) were affinity purified with m$(His)_6$VEGF-B_{186} covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia). For quantitative immunoblots, media from infected or transfected insect cells were electrophoresed together with 1–30 ng of purified m$(His)_6$VEGF-B_{186} as a standard and detected by using the affinity-purified antibodies.

Transfections, Immunoprecipitations, and Soluble Receptor Binding. 293-T cells were transfected with hVEGF$_{165}$pSG5, mVEGF-B_{167}pSG5, VEGF-B_{kEx1-5} pSG5, mVEGF-B_{186} pSG5, VEGFR-1 pIg, and VEGFR-2 pIg by using calcium phosphate precipitation. VEGFR-3 EC-Ig pREP7 (a kind gift from K. Pajusola, Biotechnology Institute, Helsinki), and hVEGF-CΔNΔC $(His)_6$pREP7 (33) were similarly expressed in 293-EBNA cells. Cells expressing the growth factors were metabolically labeled 48 hr after transfection with 100 μCi/ml Pro-mix L-[^{35}S] (Amersham) for 5–6 hr (1 μCi = 37 kBq), and the media were collected. Heparin was added to the labeling medium of VEGF-B_{167} and VEGF at 10 μg/ml, unless otherwise stated. Metabolically labeled media (except from the VEGF transfection) were immunodepleted of endogenous expressed VEGF and heterodimers by absorption for 2 hr with 2 μg/ml VEGF antibody MAB 293 on staphylococcal protein A-Sepharose. Media of the cells expressing receptor Igs were replaced 48 hr after transfection by DMEM containing 0.1% BSA and incubated for an additional 12 hr. About 50 ng of receptor-Ig fusions and the corresponding volume of media from mock-transfected cells were absorbed to protein A-Sepharose. The metabolically labeled growth factors were incubated with the receptor-Igs for 3 hr at +4°C and washed with ice-cold binding buffer (PBS/0.5% BSA/0.02% Tween 20/1 mM phenylmethylsulfonyl fluoride) three times and twice with PBS containing 1 mM phenylmethylsulfonyl fluoride. For competition studies 2 μg of recombinant hVEGF$_{165}$ was added to the binding reaction. Equal volumes of media containing the metabolically labeled factors were immunoprecipitated with the affinity-purified N-terminal peptide VEGF-B antibody, VEGF-C antiserum 882 (33), or VEGF MAB 293 for 2 hr, washed twice with ice-cold 10 mM Tris·HCl, pH 8.0/1% Triton X-100/25 mM EDTA/1 mM phenylmethylsulfonyl fluoride and twice with PBS containing 1 mM phenylmethylsulfonyl fluoride, and analyzed by SDS/PAGE.

Analysis of Competition for Binding to Cell Surface Receptors. Recombinant hVEGF$_{165}$ was labeled with Na^{125}I by using the Iodo-Gen reagent (Pierce) and purified by gel filtration on PD-10 columns (Pharmacia) to a specific activity of 1.0×10^5 cpm/ng. For binding analysis, NIH 3T3-Flt1 cells were seeded in 24-well plates coated with 0.2% gelatin, grown to confluence, washed twice with ice-cold binding buffer (DMEM/0.5 mg/ml BSA/10 mM Hepes, pH 7.4) and incubated in triplicate with 0.5 ng/ml ^{125}I-VEGF in binding buffer containing increasing amounts of unlabeled VEGF or media from VEGF-B- or mock-infected insect cells. After incubation for 2 hr at +4°C, the cells were washed three times with ice-cold binding buffer and twice with PBS containing 0.5 mg/ml BSA and lysed in 0.5 M NaOH. The solubilized radioactivity was measured with a γ counter. The nonspecific binding was less than 5% in all experiments.

Zymography and Reverse Zymography. Confluent monolayers of BME cells in 35-mm gelatin-coated tissue culture dishes were washed twice with serum-free medium, and cytokines were added in serum-free medium containing trasylol (200 Kunitz inhibitory units/ml). Fifteen hours later, cell extracts were prepared and analyzed by zymography and reverse zymography as previously described (34, 35).

RNA Preparation, *in Vitro* Transcription, and Northern Blot Hybridization. Cytokines were added to confluent monolayers of BME cells to which fresh complete medium had been added 24 hr previously. Total cellular RNA was prepared after indicated times by using Trizol reagent (Life Technologies, Basel, Switzerland). Northern blotting, UV cross-linking, methylene blue staining of filters, *in vitro* transcription, hybridization, and post-hybridization washes were as previously described (35). ^{32}P-labeled cRNA probes were prepared from bovine uPA (36), human tPA (37), and bovine PAI-1 (35) cDNAs as previously described (35, 38).

RESULTS

VEGF-B Binds Selectively to VEGFR-1. To investigate VEGF-B binding to VEGFR-1, -R-2, and -R-3, expression plasmids for mVEGF-B$_{167}$ and mVEGF-B$_{186}$ were transfected into 293-T cells. Conditioned medium from metabolically labeled transfected cells was depleted of endogenous VEGF and possible VEGF-VEGF-B heterodimers, and VEGF-B was precipitated by using VEGFR-Ig fusion proteins bound to protein A-Sepharose. Both VEGF-B splice isoforms specifically bound to VEGFR-1-Ig but not to VEGFR-2-Ig or VEGFR-3-Ig (Fig. 1*A*). The latter two receptor-Ig fusion proteins were functional as indicated by their ability to bind VEGF and VEGF-C, respectively. Interestingly, polypeptides of 32 and 16 kDa were precipitated from the mVEGF-B$_{186}$ conditioned medium with both anti-VEGF-B antibodies and by VEGFR-1-Ig. While the 32-kDa band corresponds to the glycosylated full-length form of mVEGF-B$_{186}$ (23), the 16-kDa form is likely to arise by proteolytic processing (discussed below). The binding of these two forms as well mVEGF-B$_{167}$ to VEGFR-1 was abolished by excess recombinant hVEGF (Fig. 1*B*), thus confirming the specificity of the interaction and suggesting that the receptor binding sites for VEGF and VEGF-B are, at least, partially overlapping.

We next examined the ability of VEGF-B to bind cell-surface-expressed VEGFR-1. In keeping with the data obtained with soluble receptors, conditioned medium from mVEGF-B$_{186}$ baculovirus-infected but not mock-infected

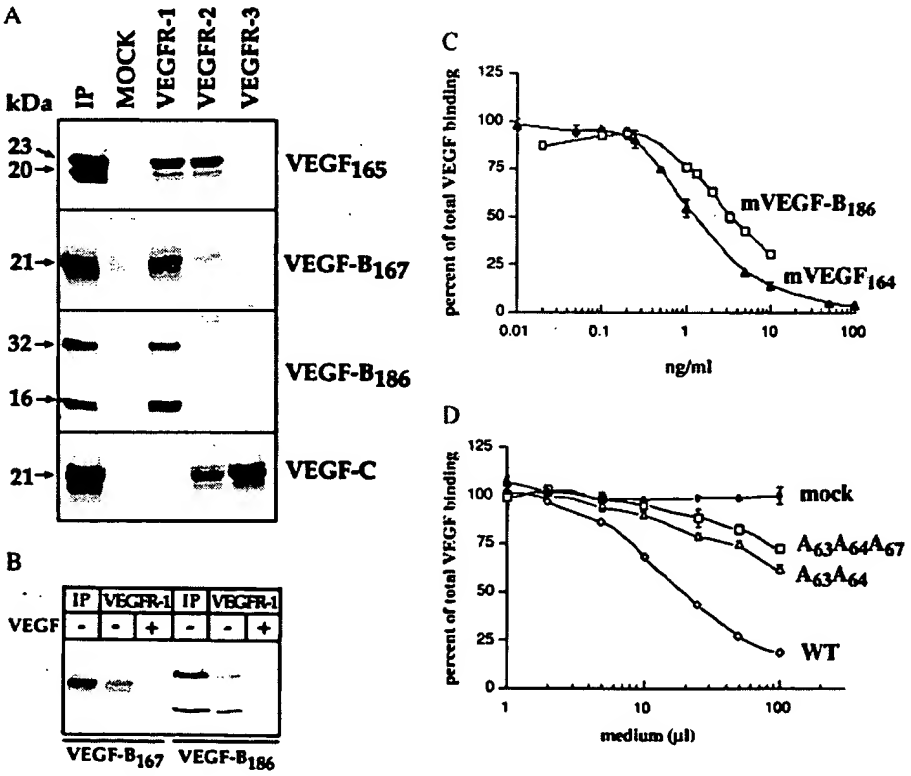

FIG. 1. VEGF-B binds selectively to VEGFR-1. (*A*) Metabolically labeled VEGF$_{165}$, mVEGF-B$_{167}$, mVEGF-B$_{186}$, or VEGF-C was precipitated by using receptor-Ig fusion proteins bound to protein A-Sepharose, or immunoprecipitated with specific antibodies as described in the text. Approximately 50 ng of each receptor-Ig was used. The precipitates were analyzed by SDS/PAGE in reducing conditions. (*B*) mVEGF-B$_{167}$ and mVEGF-B$_{186}$ were bound to VEGFR-1-Ig as above in the absence (−) or presence (+) of 2 µg of recombinant hVEGF$_{165}$. (*C*) Displacement of ^{125}I-hVEGF$_{165}$ from NIH 3T3/VEGFR-1 cells by mVEGF-B$_{186}$ or mVEGF$_{164}$. (*D*) Displacement of ^{125}I-hVEGF$_{165}$ from NIH 3T3/VEGFR-1 cells by conditioned medium containing equal amounts of mVEGF-B$_{Ex1-5}$ wild-type (WT) or mutant forms. Mean ± SD of triplicate samples in one of three independent experiment is shown.

High Five cells competed for ^{125}I-VEGF binding to NIH 3T3-Flt-1 cells (Fig. 1*C*). The half-maximum inhibitory concentration, IC_{50}, for mVEGF-B_{186} was estimated to be 3 ng/ml by quantitative immunoblotting, whereas recombinant mVEGF$_{164}$ competed for ^{125}I-hVEGF binding at an IC_{50} of 1.5 ng/ml (Fig. 1*C*).

The negatively charged amino acid residues Asp^{63}, Glu^{64}, and Glu^{67} in VEGF have been shown to be important for VEGFR-1 binding (24). To analyze whether the corresponding residues in VEGF-B are the major determinants in the VEGFR-1 interaction, Asp^{63}, Asp^{64}, and Glu^{67} were mutated to alanine residues in VEGF-B_{167} and in the truncated Ex1–5 variant. Both mutants of VEGF-B_{167} retained binding to VEGFR-1-Ig (data not shown); however, the corresponding Ex1–5 mutants showed markedly reduced receptor affinities when competing for ^{125}I-hVEGF binding to NIH 3T3-Flt-1. Interestingly, the effect of mutating all three negatively charged residues was more severe than mutations in the first two (Fig. 1*D*).

Proteolytic Processing of VEGF-B_{186}. We have previously demonstrated that mVEGF-B_{186} is modified by O-linked glycosylation, resulting in the apparent molecular mass of 32 kDa of the secreted protein (23). When mVEGF-B_{186} was expressed in 293-T cells, a 16-kDa band appeared in addition to the 32-kDa form (Fig. 1A). This band was also observed in conditioned medium from transfected COS cells labeled for a longer period. Under nonreducing conditions mVEGF-B_{186} migrated as three different dimeric polypeptides of 34, 48, and 60 kDa (Fig. 2). The 34-kDa band migrates slightly slower than the product of the mVEGF-B_{kEx1-5} construct encoded by exons 1–5, indicating that the putative cleavage site is present in the beginning of the sequence encoded by exon 6A (23). The 48-kDa band may represent a heterodimer between a processed and a full-length monomer. The three different dimeric polypeptides were able to bind VEGFR-1, and the processed subunits showed enhanced receptor binding (Fig. 2). Interestingly, plasmin treatment of unprocessed mVEGF-B_{186} expressed in COS cells resulted in an N-terminal 15-kDa fragment capable of interacting with VEGFR-1-Ig (data not shown). This finding suggested that, similarly to VEGF, the plasmin cleavage product contains the receptor-binding epitopes.

Mutational Analysis of Conserved Cysteine Residues. To examine the contribution of the conserved cysteine residues for VEGF-B dimerization, Cys^{51} (Cys 2) and Cys^{60} (Cys 4) were mutated to serine residues individually (C2S and C4S) or in combination (C2S,C4S) (Fig. 3*A*). The mutants and wild-type VEGF-B_{167} were either immunoprecipitated with the affinity-purified N-terminal VEGF-B antibody and analyzed under nonreducing and reducing conditions, or bound to VEGFR-1-Ig (Fig. 3*B*). All mutants were expressed in approximately

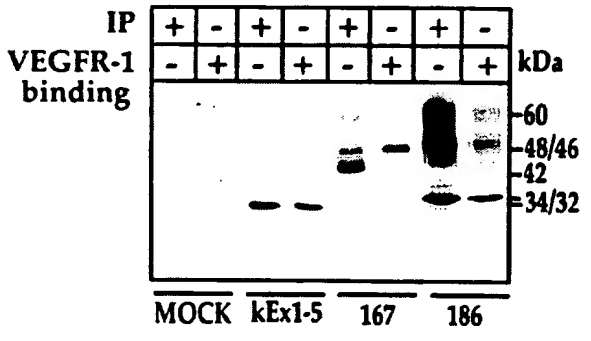

FIG. 2. VEGF-B_{186} is proteolytically processed. Metabolically labeled VEGF-B_{kEx1-5}, VEGF-B_{167}, and VEGF-B_{186} were immunoprecipitated (IP) with the affinity-purified N-terminal VEGF-B antibody or bound to VEGFR-1-Ig. The bound ligands were analyzed by SDS/PAGE in nonreducing conditions.

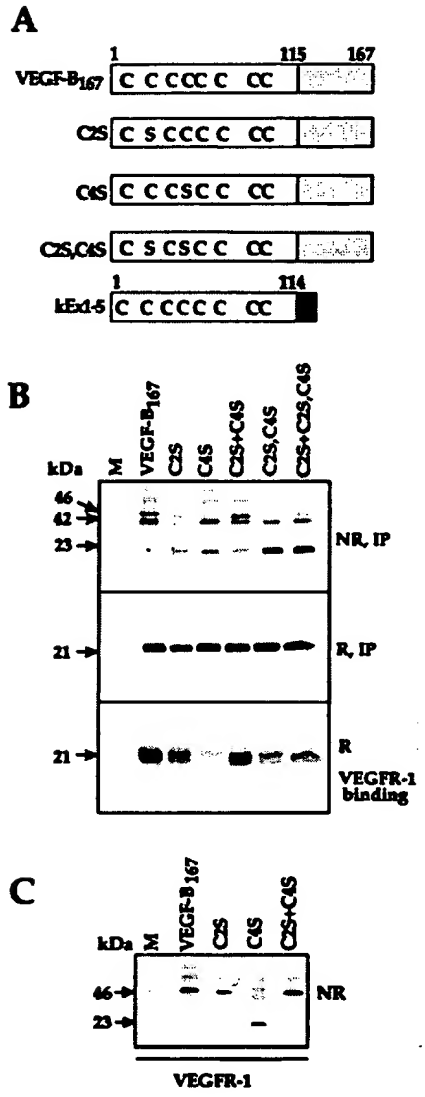

FIG. 3. Mutational analysis of conserved cysteine residues in VEGF-B_{167}. (*A*) Schematic illustration of wild-type and mutant forms of VEGF-B_{167}. (*B*) The cysteine mutants were transfected individually or in combination, labeled in the presence of 50 μg/ml heparin, subjected to immunoprecipitation (IP) or VEGFR-1 Ig binding, and analyzed by SDS/PAGE in reducing (R) and nonreducing (NR) conditions. (*C*) Some of the VEGF-B_{167} mutants were expressed as above and labeled in the presence of 10 μg/ml heparin. The conditioned media were incubated with VEGFR-1-Ig and the bound ligands were subjected to SDS/PAGE under nonreducing conditions.

similar amounts (Fig. 3*B Middle*). Wild-type VEGF-B_{167} migrated under nonreducing conditions as two bands, 42 and 46 kDa; however, only the 46-kDa form bound to VEGFR-1-Ig (see Figs. 2 and 3 *B* and *C*). The 42-kDa band may correspond to dimers joined together by aberrant disulfide bonding, since such a polypeptide doublet is not seen in VEGF-B_{186} or VEGF-B_{kEx1-5}, which lack the additional eight cysteine residues found in the C-terminal part of VEGF-B_{167}. The mutant C4S gave rise to monomers and some dimers migrating at 42 kDa, which were unable to bind to VEGFR-1-Ig. Surprisingly, the C2S mutant, although partially monomeric, could still form dimers capable of receptor binding. Cotransfection of the single mutants (C2S+C4S) resulted in increased amounts of the receptor-binding 46-kDa form, indicating that they complement each other by establishing a disulfide link between the nonmutated cysteine residues, probably in the same way as

shown for VEGF (25). Cotransfection of a single mutant with the double mutant failed to complement. However, both C4S and C2S,C4S showed residual receptor binding (Fig. 3*C*), which could be explained by the interaction of VEGFR-1-Ig with the monomers.

VEGF-B Increases uPA and PAI-1 Synthesis. The competition analysis using purified recombinant $(His)_6VEGF\text{-}B_{186}$ indicated that only a minor portion of the protein is biologically active, since the native unpurified $VEGF\text{-}B_{186}$ competed more effectively with ^{125}I-hVEGF for binding to VEGFR-1-expressing cells. Despite this, we tried to address some of the biological responses to VEGF-B. By Northern blot analysis, we found that $VEGF\text{-}B_{186}$ (50 ng/ml) increased the steady-state levels of uPA and PAI-1 mRNAs in BME cells (Fig. 4*A*). The kinetics of PAI-1 induction was more rapid and transient than of uPA, in agreement with findings reported for bFGF and VEGF (11, 35, 39). $VEGF\text{-}B_{186}$ also induced PAI-1 but not uPA mRNA in bovine aortic endothelial cells which, like BME cells, express VEGFR-1 as judged by reverse transcriptase-coupled PCR analysis using specific primers (data not shown). By zymography and reverse zymography, $VEGF\text{-}B_{186}$ induced a dose-dependent increase in uPA and PAI-1 activity in BME cells (Fig. 4*B*). In contrast to VEGF (11), $VEGF\text{-}B_{186}$ did not increase tPA activity. The apparent lack of induction of PAI-1 activity by VEGF, used as a control, may reflect the rapid sequestration of PAI-1 into a complex with VEGF-induced tPA; this complex is observed by zymography of the culture supernatant of VEGF-treated cells (data not shown).

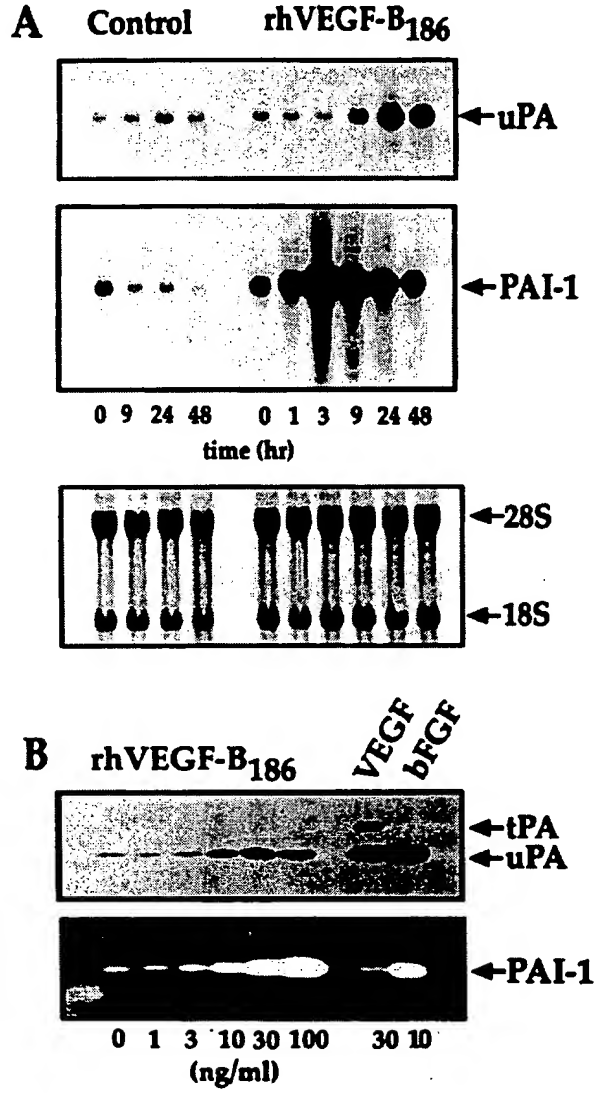

FIG. 4. Recombinant $hVEGF\text{-}B_{186}$ increases the expression and activity of uPA and PAI-1 in BME cells. (*A*) Replicate filters, containing 5 μg per lane of total cellular RNA prepared from confluent monolayers of BME cells incubated in the presence of 50 ng/ml $hVEGF\text{-}B_{186}$ for the indicated times, were hybridized with ^{32}P-labeled cRNA probes as described in the text. RNA integrity and uniformity of loading were determined by staining the filters with methylene blue after transfer and cross-linking (*Bottom*); 28S and 18S ribosomal RNAs are shown. (*B*) Cell extracts prepared from BME cells, incubated in the presence of $hVEGF\text{-}B_{186}$, VEGF, or bFGF at the indicated concentrations for 15 hr, were subjected to zymography (*Upper*) and reverse zymography (*Lower*) as described in the text.

DISCUSSION

In this study we demonstrate that VEGF-B specifically binds to VEGFR-1 and that endothelial cells respond to VEGF-B by increased expression and activity of uPA and PAI-1. Mutations of the conserved cysteine residues indicate that VEGF-B forms, similarly to VEGF, antiparallel covalent dimers. We also show that $VEGF\text{-}B_{186}$ is proteolytically cleaved, resulting in an N-terminal fragment, which contains the cystine knot motif as well as the receptor-binding epitopes.

VEGF-B is the third ligand identified for VEGFR-1, the others being VEGF and PlGF. The VEGF-binding determinant has been localized to the second Ig-like domain on VEGFR-1, whereas full VEGF binding requires the context of the first and especially the third Ig-like domain (40–42). The first three Ig-like domains of VEGFR-1 are also sufficient for VEGF-B binding (unpublished data), which is in agreement with the finding that VEGF and VEGF-B compete for VEGFR-1 binding. Charged amino acid to alanine scan mutagenesis led Keyt *et al.* (24) t propose that the VEGFR-1-binding epitope in VEGF involves a stretch of acidic residues (Asp^{63}, Glu^{64}, and Glu^{67}) that are located in loop II. These acidic amino acid residues are conserved in VEGF-B and partially also in PlGF. Our data indicate that these residues are contributing to the receptor affinity; however, they might not be the major determinants for VEGFR-1 binding. While this manuscript was in preparation, the crystal structure of VEGF in complex with the second Ig-homology domain f VEGFR-1 was reported (42). The VEGF–VEGFR-1 domain 2 interface is dominated by hydrophobic contacts, and many VEGF residues found to be important for VEGFR-2 binding (26) are also buried in the interface with VEGFR-1 (42). Asp^{63} is involved in a polar interaction, whereas Glu^{64} and Ile^{43} may be in contact with the third domain of VEGFR-1. Further studies are needed to elucidate the amino acid residues in VEGF-B critical for VEGFR-1 binding.

VEGF-B belongs to a growth factor superfamily containing a cystine knot motif. In addition to the disulfide bridges in the cystine knot in VEGF-B, two disulfide bridges join the two antiparallel monomers into a dimer. When Cys^{51} and Cys^{60} were mutated into serine residues, VEGF-B dimer formation and VEGFR-1 binding were severely reduced. Coexpression f the single mutants complemented the dimerization and receptor-binding defects.

The observed proteolytic processing of $VEGF\text{-}B_{186}$ may regulate receptor affinity, but is perhaps not important for bioavailability of the protein, since this isoform is readily secreted from cells. However, the apparently increased affinity of the processed form needs to be confirmed by using recombinant $VEGF\text{-}B_{186}$ forms. Recently, VEGF-C was shown to undergo proteolysis, with trimming of both N- and C-terminal extensions of VEGF-C, which resulted in an increased affinity for VEGFR-3 and activation of a VEGFR-2-binding property (33). Also the generation of an active fragment of $VEGF_{189}$ has been reported t require proteolysis (43).

VEGF and VEGF-B are likely to have nly partially overlapping biological roles due to their different recept r binding

specificities. Results of gene targeting experiments have indicated that VEGFR-2 is required for the development of endothelial cells (7), and VEGFR-1 plays a role in vascular organization (6). Functional differences between the two VEGFRs have been observed also when receptor-transfected cells were used: whereas VEGFR-2-expressing fibroblasts respond to VEGF by proliferation (44), VEGFR-1-expressing cells fail to do so (45). In keeping with this observation, we have not been able to demonstrate any mitogenic effects by using purified recombinant tagged VEGF-B, although one cannot exclude the possibility that the amount of active protein in these preparations is not sufficient to trigger a response. It is possible that the previously reported stimulation of DNA synthesis by VEGF-B_{167} conditioned medium from 293-EBNA cells (15) is due to heterodimer formation with the endogenous VEGF, similar to what has been reported for PlGF-VEGF heterodimers (46, 47). It is also possible that the binding of VEGF-B to VEGFR-1 enables VEGF to bind primarily to VEGFR-2 and to elicit a mitogenic response. In fact, PlGF has been shown to potentiate the activity of low concentrations of VEGF (21).

In this report we show that endothelial cells respond to VEGF-B by inducing the expression and activity of uPA and PAI-1. Interestingly, PAI-1 expression preceded that of uPA by several hours, similar to results with VEGF (11, 39). The concerted expression of PAI-1 and uPA may serve to protect the ECM from extensive proteolysis. Alternatively, PAI-1 may function independently of its role as an inhibitor. With regard to this latter possibility, it is of interest that PAI-1 has been shown to compete with integrin $\alpha_v\beta_3$ for vitronectin binding (48), enabling it to regulate cell–matrix interactions. PAI-1 and uPA may thus regulate both proteolysis and cellular migration during different stages of angiogenesis, and these dual functions are likely to be synergistic.

In this study we have identified VEGF-B as the second VEGFR-1-specific ligand. VEGF-B has a broad tissue expression pattern as opposed to PlGF, suggesting that it might be a functional homologue of PlGF in tissues others than the placenta. It might be involved in endothelial ECM degradation and adhesion, but *in vivo* studies will be necessary to characterize the physiological role of VEGF-B in angiogenesis and vascular maintenance.

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